PHYSIOLOGIA PLANTARUM

VOL. IX

1956

ED.

SOCIETAS PHYSIOLOGIAE PLANTARUM
SCANDINAVICA

The pages 172-176 have not been published.

Index

Fasc. 1

Index auctorum

Determination of Photothermic and Vernalization Quanta for the Vegetative Period of Wheat

By

J. J. Chinoy

University of Delhi, Delhi-8 (India)
(Received July 7, 1955)

Klebs's (1918) postulate of the condition of 'ripeness to flower' in plants, Gassner's (1918) concept of a specific 'cold requirement' of winter cereals, and the photoperiodic studies of Garner and Allard (1920) paved the way for Lysenko's theory of phasic development. Garner and Allard's concept of photoperiodism is now no longer tenable in the light of the increasing evidence that the plants require a certain amount of light (or darkness) for flowering and not necessarily a diurnal alternation of light and dark periods (Lysenko, 1935; Hamner, 1944; Chinoy and Nanda, 1951 a, 1951 b, 1951 c, 1952).

There is also increasing evidence to show that the photoperiodic response of a plant is greatly dependent upon temperature. The influence of temperature upon the photophase and subsequent phases is recognised by Dolgŭsin and Lysenko (1929). They have shown that the higher the temperature of the growing period the earlier will be the shooting in winter cereals, provided that they are vernalized. On the basis of the above mentioned fact it would follow that the effect of temperature on flowering could be separated into two categories: (1) the effect of temperature on the thermophase of a plant, and (2) the effect of temperature on the subsequent phases of a plant. In this connection the studies of Purvis (1934) are interesting because they clearly indicate an interrelation of day length with temperature of germination in winter rye. From his studies of vegetative periods of a large number of barley varieties, grown at 30 different places having a wide range of climatic conditions, Bakhteyev (1935) has also concluded that "a

[1]

long day is effective in shortening the period before heading out only in presence of a certain temperature".

Further, Purvis and Gregory (1937) have shown that low temperature treatment (vernalization) can be substituted by short day treatment during germination of winter rye for accelerating its flowering. Voss (1938) could also induce shooting in German winter wheats by short day treatment of germinating seed kept at approximately 20° C. These findings are suggestive of the fact that even for the completion of the thermophase a relationship exists between the effects of temperature and photoperiod. Hamner (1938) and Thompson (1939) have rightly stressed the need for studying the inter-relations of factors, especially the relation between temperatures and photo-periodic responses of a plant. The interrelationship between the effects of temperature and photoperiod for the initiation of flowering in a number of so-called long and short-day plants have also been clearly demonstrated by a number of other workers (Naylor, 1941; Rudorf and Schroeck, 1941; Borthwick et al., 1941—42, 1943; Sakr 1944).

The phenomenon of thermoperiodicity demonstrated by Went (1944, 1945, 1948) also clearly indicates that the photoperiodic response of a plant is determined by the temperature of the growing season and is varied at differ-ent temperatures. Lang and Melchers (1943) have also shown in the case of Hyoscyamus that the photoperiodic response of this plant depends upon temperature so much so that the critical length of the day increases with temperature.

During the course of work on correlations between growth and the time of flowering of wheat varieties (Chinoy, 1949), and their responses to ver-nalization and photoperiodic treatments (Chinoy, 1949 a, 1950) it was ob-served that any alteration in the rate of the developmental process changed the growth pattern of the plant. The photoperiodic responses of different varieties of wheat appeared to be correlated with the length of the vegeta-tive period, thus suggesting a quantative relationship between the amount of energy utilized by the plant and some of its major physiological processes. This was particularly noticeable in experiments in which a graded series of photoperiodic treatments were given by placing the plants either in con-tinuous light or in reduced photoperiod for 5, 10, 15, 20, 25, 30, 35, and 40 days respectively, besides growing them in normal conditions of light (Chinoy, 1950).

Attempts were, therefore, made to determine the amount of energy received by different varieties of wheat belonging to different flowering classes during their vegetative periods, when subjected to vernalization and photoperiodic treatments. In the present communication results of three such complex

experiments involving different combinations of varieties, vernalization and light treatments are presented.

Experimental Procedure

Varieties: In Experiment I (partly reported previously — Chinoy, 1950) only one variety of wheat, Viz., N.P. 52 (Triticum vulgare) was used. The range of variation in lengths of vegetative periods of the seven varieties used for Experiment II was not very wide (80 to 105 days). All the seven varieties belonged to Triticum vulgare; and four of them were Indian and three were exotic varieties. The Indian varieties were: N.P. 165, N.P. 52, Punjab C. 591, and Punjab 8-A; and the exotic varieties were Cadia, Union, and Carlisle. In Experiment III the range of variation in the lengths of the vegetative periods of the twelve varieties used was very wide (99 to 162 days). The twelve varieties were numbered serially in the order of the increasing lengths of their vegetative periods of plants raised from unvernalized seed and grown under normal day treatment as follows:

(1) Triticum vulgare, var. N.P. 165; (2) T. dicoccum, var. Khapli; (3) T. vulgare, var. Buhi; (4) T. durum, var. Mindum; (5) T. durum, var. Kubanka; (6) T. vulgare, var. Acme; (7) T. persicum, var. Persian Black; (8) T. vulgare, var. Amantka; (9) T. polonicum, var. Polish Wheat; (10) T. vulgare, var. Yeoman II; (11) T. vavilo-vianum, var. Vaneum Yakub; and (12) T. turgidum, var. Cambridge Rivett.

Vernalization treatment was given in the case of Experiments II and III. Graded seeds were soaked for two hours in distilled water and then placed in Petri dishes (15 cm. in diameter) on a sterilized filter paper with a thin layer of sterile sand and 7.0 ml. of distilled water were added to each and after covering them were allowed to stand at room temperature ($30—20° $ C) for 20 hours during which the seed coat ruptured and coleorhiza emerged. After the initiation of germination all seeds which failed to germinate were removed leaving 150 seeds in each Petri dish. 30 dishes were kept for each variety. The seeds were then covered up with equal quantities of sterile sand, moistened with 25 ml. of distilled water and placed in a refrigerator at $3—5°$ C. This method of keeping the seeds for vernalization en-sured very uniform response to the treatment on account of the very even distribu-tion of moisture. Covering the seeds with sterile sand completely eliminated fungal infection, which could not be entirely avoided if left uncovered even after the sterilization of seed. No attempt was made to restrict growth of seedlings. In fact the growth of the coleoptile appeared to bring about complete vernalization in a shorter period of time as compared to the germinated seed in which growth was inhibited by restricting watering when kept at the same temperature (3 -$5°$ C). Moisture of the Petri dishes was replenished whenever necessary by the addition of distilled water. Periods of vernalization in Experiment II and III were of 38 and 45 days respectively.

Five days before the end of vernalization treatment another batch of seeds were germinated at room temperature ($27—20°$ C). After this control (C) set had attained the same growth stage as the vernalized (V) one, seedlings of both the sets were transplanted in 12 inch unglazed earthen pots, containing 3 to 1 mixture of field soil and well rotted farm yard manure in Experiments II and III. In the case of Experiment I 10 seeds were sown directly in each pot. Five pots each with

seven seedlings were used for each treatment in Experiments I and II; and eight pots each with five seedlings were used for Experiment III.

Manuring and watering: Two days after germination in Experiment I and transplantation in Experiments II and III the pots were manured with Nicifos and potassium sulphate, 2 and 1 g. respectively per pot. This fertilizer dose was repeated every week for eight weeks. The pots were adequately watered every day except during December and January, when watering on alternate days was found to be adequate.

Photoperiodic Treatments: Immediately after germination in Expt. I, ten days after transplantation in Expt. II and on the first day of transplantation in Experiment III the pots were transferred to different light treatments. The arrangement for giving long and short day treatments has already been shown elsewhere (Chinoy and Nanda, 1951 a). In Experiment I there were eight short day treatments of 40, 35, 30, 25, 20, 15, 10, and 5 short (6-hour) days, a normal day treatment with mean daily photoperiod of 11 hours, and eight long (24-hour) day treatments of 5, 10, 15, 20, 25, 30, 35, 40 days respectively; and in Expt. II there were three short (7-hour) day treatments of 45, 30, and 15 short (7-hour) days, a normal day treatment with mean daily photoperiod of 11 hours, and three long (24-hours) day treatments of 15, 30, and 45 days respectively. In Experiment III there were three light treatments. viz., short (6-hour) day treatment of 85 days, a normal day treatment with mean daily photoperiod of 11 hours throughout course of the experiment, and one long day (24-hour day) treatment throughout its vegetative period. The seventeen photoperiodic treatments as given in the above increasing order of the amount of light have been designated in the text for Expt. I as 40-SD, 35-SD, 30-SD, 25-SD, 20-SD, 15-SD, 10-SD, 5-SD, ND, 5-LD, 10-LD, 15-LD, 20-LD, 25-LD, 30-LD, 35-LD, 40-LD; the seven photoperiodic treatments of Expt. II as 45-SD, 30-SD, 15-SD, ND, 15-LD, 30-LD, and 45-LD; and the three photoperiodic treatments of Expt. III as SD, ND, and LD treatments respectively. In all photoperiodic treatments where a definite number of short or long days were given the remaining days of the vegetative period were normal (11-hour) days.

Vegetative periods: Vegetative periods were determined by noting the dates of anthesis individually for 10, 25, and 24 plants for each treatment combination of Expts. I, II, and III respectively. Periods in days between the dates of sowing or transplantation and anthesis of each of these plants were determined and from this data mean vegetative periods for a given variety under different vernalization and photoperiodic treatments were obtained. The method of noting the morphological change in the growing points (double ridges), suggested by Purvis (1934) was not practicable for experiments of such magnitudes.

Photo Quantum: Total number of light hours received by the plants during the vegetative periods under different treatment combinations were calculated individually for all the observations from the diurnal records of hours of natural light and adding to that the total number of light hours of short or long days in each treatment. The sum of light hours during the vegetative period of a variety is described in this paper as its *photo quantum* (P).

In order to eliminate as far as possible, differences in intensities of light incident upon different pots receiving artificial illumination, they were shifted every third day in such a manner that those pots which were nearer to the sources of light for two nights were removed to positions occupied by those pots which were further

away and vice versa. Daily records of intensities of natural light were obtained by means of direct reading Weston Illumination Meter — Model 756 (Quartz Filter), manufactured by Weston Electrical Instrument Corporation, Newark 5, New Jersey, U.S.A., three times a day throughout the growing period and mean intensities were calculated. Mean intensity of artificial light was also obtained by determining the intensities at measured distances from the lamps and taking a mean of a large number of readings. Intensities of illuminations have, however, not been taken into consideration in this paper as it is proposed to deal with the influence of light intensities more fully in a subsequent communication.

Temperature records: Diurnal records of maximum and minimum temperatures were kept throughout the season and daily means of temperatures were worked out. The sum of the daily mean temperatures during the vegetative period of a variety under any given treatment is described in this paper as its *thermic quantum* (T).

The values of Photo- and Thermic-Quanta entered in the text are to be multiplied by (10^3) in some cases (e.g. Figure 1).

Experimental Findings

The data of mean photo quanta for each of the seventeen light treatments of Expt. (I) have been plotted against the corresponding mean thermic quanta of plants of N.P. 52 in Figure 1. A highly significant inverse correlation was found to exist between the photo and the thermic quanta. Regression of the photo quantum on the thermic quantum was calculated by the method of linear regression using the logarithms of the photo and the thermic quanta for the analysis. From the regression equation so derived the position of the curve in Figure 1 was determined by plotting the statistically calculated values of photo quanta from the equation against the thermic quanta. It will be seen that the experimental values of photo quanta are in close agreement with the statistical ones.

Photothermic quantum: The products of the corresponding values of photo and thermic quanta of a variety under different photoperiodic treatments gave values which varied within a certain range of experimental error. This product of photo and thermic quanta has been designated as the *photo-thermic quantum* (E) of a variety in this paper.

Vegetative periods of plants under seventeen light treatments in Expt. I together with the corresponding experimental and statistical values of the photothermic quanta are given in Table 1. Experimental values have been obtained by multiplying the values of photo quanta by the corresponding thermic quanta and taking their mean. Statistical values have been obtained by multiplying the values of photo quanta calculated from the regression equation with the corresponding experimental values of the thermic quanta. There is fairly good agreement between the experimental and the statistical

FIGURE I

$LOG\ Y = 7.451\ \ -1.169\ \ (LOG\ X)$

PHOTO QUANTUM (P) = Y.10³

THERMIC QUANTUM (T) = X.10³
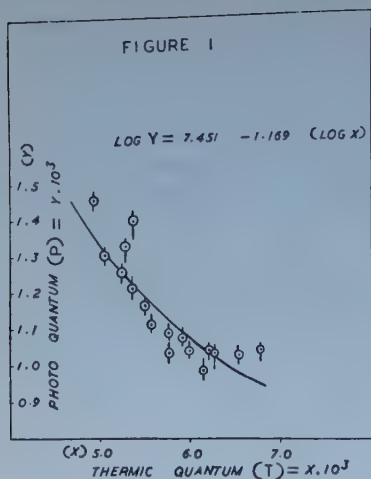
Figure 1. *The relation between the photo quanta and the thermic quanta of Experiment I.*

values of photothermic quanta inspite of the fact that adequate control of light intensities and temperatures of the growing season could not be achieved.

Having determined the photothermic quantum of one wheat variety it was considered of interest to investigate the relationship between the vegetative periods of wheat varieties belonging to different classes of flowering and their photothermic quanta. An attempt was also made to determine the effect of vernalization on the photothermic quantum of a variety. Accordingly in Experiment II photothermic quanta of seven varieties of wheat were determined under differential photoperiodic and vernalization treatments. The logarithms of the photo quanta for unvernalized and vernalized plants of all the seven varieties in Expt. II have been plotted against the logarithms of the corresponding thermic quanta in Figure 2. Regression of photo quantum on thermic quantum was calculated separately for unvernalized and vernalized plants of each of the seven varieties and positions of trend lines were determined from regression equations given in Table 2. In all cases the experimental values correspond closely to trend lines (Figure 2).

It is evident from the lower values of photothermic quanta for the vernalized plants that the efficiency of the utilization of photothermic energy, or in other words the photosensitivity of the plant, increases with vernalization. The difference between the photothermic quantum of the unvernalized and the vernalized plants has been considered as the *Vernalization Quantum* of a variety. Vegetative periods of plants under fourteen combinations of photoperiodic and vernalization treatments for seven varieties of Experiment II, together with the corresponding experimental and statistical values of the photothermic and the vernalization Quanta are presented in Figure 3. Agree-

Table 1. *Vegetative Periods and Experimental and Statistical Values of Photothermic Quanta of Triticum vulgare, var. N.P. 52 under seventeen different photoperiodic treatments.* Regression Equation: Log Y=7.451—1.169 (Log x).

Light Treatment	Vegetative Period (Days)	Photothermic Quantum	
		Experimental Values	Statistical Values
40-LD	85.0	7.17	6.70
35-LD	87.0	6.58	6.67
30-LD	91.5	7.49	6.61
25-LD	91.0	7.02	6.62
20-LD	90.5	6.58	6.63
15-LD	92.0	6.48	6.61
10-LD	93.5	6.38	6.58
5-LD	95.0	6.18	6.57
ND	98.0	6.23	6.52
5-SD	99.5	6.34	6.50
10-SD	98.0	5.94	6.52
15-SD	101.0	6.21	6.48
20-SD	103.5	6.46	6.44
25-SD	105.0	6.47	6.43
30-SD	103.0	6.04	6.46
35-SD	109.0	6.70	6.39
40-SD	113.0	7.06	6.35

Each value is a mean of ten readings.

Multiply the values given in the table by 10^6 to obtain the values of photothermic and vernalization Quanta.

ments between experimental and statistical values of photothermic and vernalization quanta are good in all cases. Another point worthy of note is that the value of photothermic quantum increases with the length of the vegetative period of a variety and this relationship between the length of the vegetative period and the magnitude of the photothermic quantum is maintained for unvernalized plants of all the seven varieties under all the seven light treatments as seen from Figure 3. It is also interesting to note that the longer the vegetative period of a variety the greater is the vernalization quantum.

Mean values of vegetative periods for unvernalized and vernalized plants of the seven varieties, together with corresponding means of experimentally and statistically determined photothermic and vernalization quanta and the regression equations are given in Table 2. The results clearly demonstrate the relationship between the vegetative period of a variety and its photothermic and vernalization quanta.

A similar analysis was carried out on the data of Experiment III. Regression of photo quanta on thermic quanta for unvernalized and vernalized plants of the 12 varieties of wheat with a much wider range of flowering were calculated individually and positions of trend lines were determined.
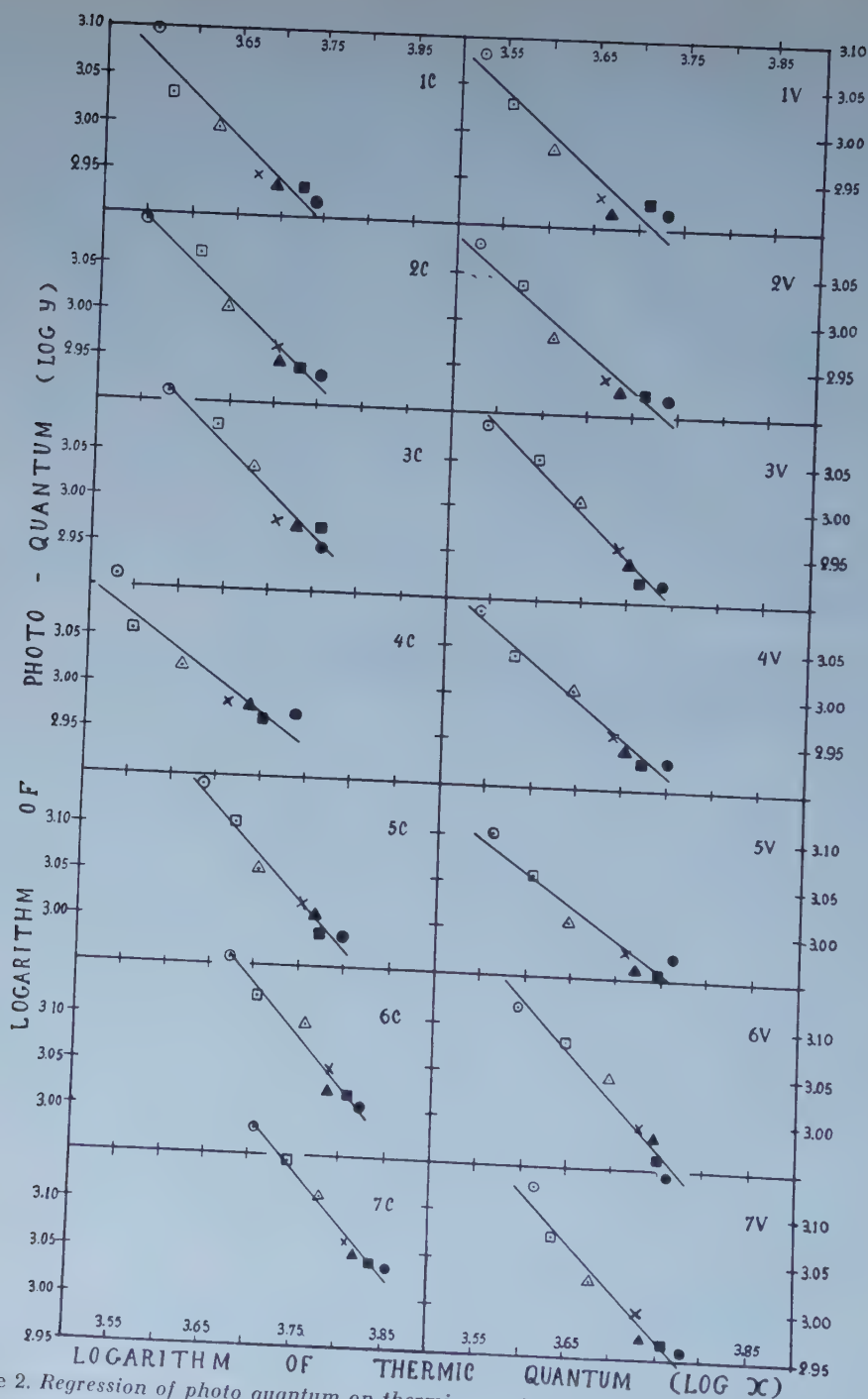
Figure 2. *Regression of photo quantum on thermic quantum of unvernalized and vernalized plants of the seven varieties of wheat* (Experiment II). 1 C to 7 C are unvernalized plants, and 1 V to 7 V are vernalized Plants of the seven varieties in the serial order given in Table 2. 45-LD=⊙; 30-LD=⊡; 15-LD=△; ND=×; 15-SD=▲; 30-SD=■; 45-SD=●.

Table 2. *Mean vegetative periods, regression equations, and mean values of experimentally and statistically determined photothermic and vernalization quanta for unvernalized and vernalized plants of seven varieties of wheat.*

Serial No.	Variety	Vegetative Period Days		Regression Equation	Photothermic Quantum		Vernalization Quantum	
					Experimental Values	Statistical	Experimental Values	Statistical
1	N.P. 165	(C)	77.3	Log Y=6.422—0.942 Log x	4.31	4.30		
1	N.P. 165	(V)	74.6	Log Y=6.164—0.879 Log x	4.07	4.02	0.24	0.27
2	N.P. 52	(C)	79.5	Log Y=6.421—0.935 Log x	4.55	4.56		
2	N.P. 52	(V)	76.0	Log Y=5.944—0.815 Log x	4.16	4.16	0.38	0.41
3	Punjab C. 591	(C)	84.0	Log Y=6.524—0.951 Log x	5.07	5.07		
3	Punjab C. 591	(V)	78.7	Log Y=6.455—0.947 Log x	4.45	4.45	0.62	0.61
4	Cadia	(C)	82.8	Log Y=5.665—0.721 Log x	4.95	4.94		
4	Cadia	(V)	78.5	Log Y=5.908—0.798 Log x	4.45	4.45	0.50	0.51
5	Punjab 8 A .	(C)	90.2	Log Y=7.018—1.067 Log x	5.87	5.87		
5	Punjab 8 A .	(V)	81.7	Log Y=5.578—0.699 Log x	4.81	4.85	1.06	1.01
6	Union	(C)	96.0	Log Y=7.084—1.069 Log x	6.68	6.68		
6	Union	(V)	85.9	Log Y=5.306—1.033 Log x	5.31	5.30	1.38	1.38
7	Carlisle	(C)	102.0	Log Y=7.077—1.052 Log x	7.58	7.58		
7	Carlisle	(V)	84.5	Log Y=6.789—1.017 Log x	5.32	5.32	2.26	2.26

(C) — unvernalized, and (V) — vernalized plants.

Multiply the values given in the Table by 10^6 to obtain the values of photothermic and vernalization quanta.

The relationship between the photo and the thermic quanta of different varieties was found to be the same as in the first two experiments, and therefore, for the sake of brevity of presentation of the data these regression graphs are not included in this paper. However, in Figure 4 experimentally determined values of photo and thermic quanta of the twelve varieties are plotted against lengths of vegetative periods separately for the six treatment combinations of light and temperature of germination (vernalization). Also mean vegetative periods of the twelve varieties, mean experimental and statistical values of photothermic and vernalization quanta, as well as the regression equations are presented in Table 3. There is a progressive increase in the photo and thermic quanta of wheat varieties (Figure 4) with the length of their vegetative periods. These varieties are serially numbered 1 to 12 in an increasing order of their lengths of vegetative periods. This relationship is found to hold good for unvernalized plants under all the three light treatments LD.C, ND.C, and SD.C of Figure 4. For vernalized plants (LD.V, ND.V, and SD.V of Figure 4) also this relationship holds good for all the varieties except for the three winter varieties (Nos. 10, 11 and 12) which show interactions between photoperiodic and vernalization treatments. Another point worthy of note is that although both the photo (P) and the

Figure 3. *Vegetative periods* (in figures) *of plants under fourteen combinations of photo-periodic and vernalization treatments for seven varieties of wheat* (Experiment II), *together with the corresponding experimental and statistical values of the photothermic and the vernalization quanta.*
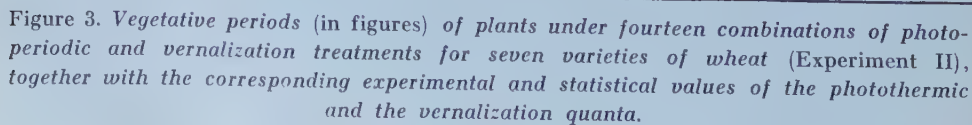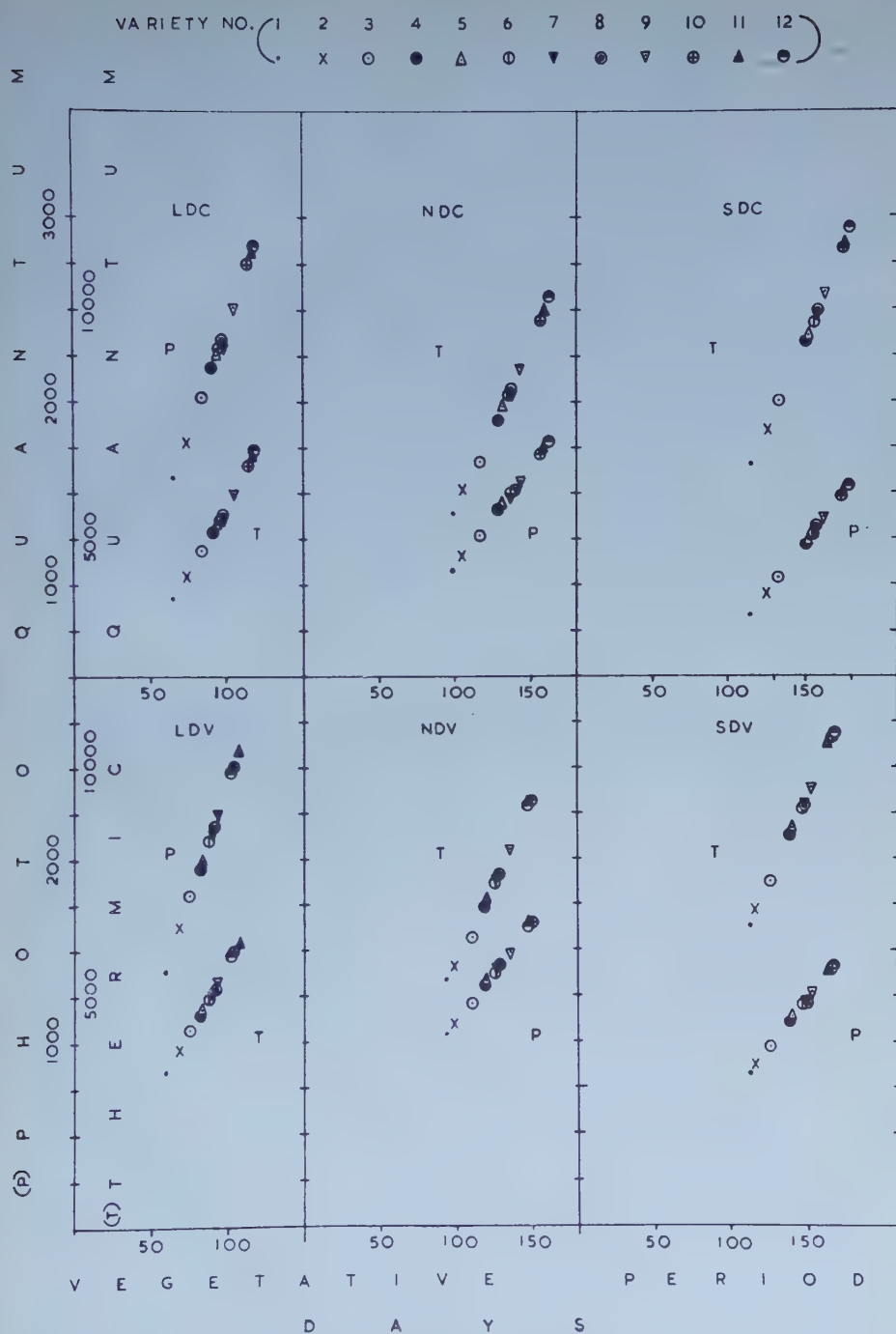
Figure 4. *The relationship between the length of the vegetative period and the photo and the thermic quanta of unvernalized and vernalized plants under three light theatments of twelve varieties of wheat in Experiment III.*

Table 3. *Mean vegetative periods, regression equations, and mean experimental and statistical values of photothermic and vernalization quanta of twelve varieties of wheat.*

Serial No.	Variety		Vegetative Period (Days)	Regression Equation	Photothermic Quantum		Vernalization Quantum	
					Experimental Values	Statistical Values	Experimental Values	Statistical Values
1	N.P. 165	(C)	92.9	Log Y=7.047—1.075 Log x	5.85	5.84		
1	N.P. 165	(V)	88.8	Log Y=5.965—0.791 Log x	5.22	5.45	0.62	0.39
2	Khapli	(C)	101.3	Log Y=7.760—1.240 Log x	7.21	7.20		
2	Khapli	(V)	94.8	Log Y=7.076—1.076 Log x	6.15	6.19	1.06	1.06
3	Buhi	(C)	111.2	Log Y=8.038—1.287 Log x	8.88	8.88		
3	Buhi	(V)	104.0	Log Y=7.270—1.104 Log x	7.53	7.53	1.36	1.35
4	Mindum	(C)	123.7	Log Y=6.892—0.959 Log x	11.28	11.28		
4	Mindum	(V)	115.3	Log Y=6.756—0.941 Log x	9.57	9.56	1.72	1.71
5	Kubanka	(C)	125.6	Log Y=7.503—1.113 Log x	11.69	11.69		
5	Kubanka	(V)	113.3	Log Y=6.698—0.930 Log x	9.18	9.18	2.51	2.51
6	Acme	(C)	129.5	Log Y=7.015—0.979 Log x	12.54	12.54		
6	Acme	(V)	122.0	Log Y=6.909—0.967 Log x	10.92	10.91	1.62	1.62
7	Persian Black ..	(C)	129.4	Log Y=7.413—1.081 Log x	12.54	12.54		
7	Persian Black ..	(V)	120.5	Log Y=6.775—0.935 Log x	10.58	10.58	1.96	1.96
8	Amantka	(C)	130.3	Log Y=6.891—0.945 Log x	12.72	12.72		
8	Amantka	(V)	122.1	Log Y=7.248—1.054 Log x	10.94	10.94	1.78	1.78
9	Polish Wheat ..	(C)	137.1	Log Y=7.719—1.143 Log x	14.41	14.40		
9	Polish Wheat ..	(V)	128.0	Log Y=7.026—0.985 Log x	12.18	12.18	2.23	2.22
10	Yeoman II	(C)	148.2	Log Y=7.719—1.143 Log x	17.32	17.32		
10	Yeoman II	(V)	140.0	Log Y=7.911—1.170 Log x	15.10	15.10	2.22	2.22
11	Vaneum Yakub .	(C)	151.2	Log Y=8.231—1.247 Log x	17.91	17.91		
11	Vaneum Yakub .	(V)	140.2	Log Y=7.889—1.181 Log x	15.17	15.16	2.75	2.75
12	Cambridge Rivett	(C)	152.9	Log Y=7.955—1.172 Log x	18.78	18.78		
12	Cambridge Rivett	(V)	139.2	Log Y=7.011—0.959 Log x	14.83	14.84	3.94	3.94

(C) — unvernalized, and (V) — vernalized plants.

Multiply the values given in the Table by 10^6 to obtain the values of photothermic and vernalization quanta.

thermic (T) quanta increase with the length of the vegetative period of a variety these values for any given variety are inversely correlated with each other; thus for example, under LD.C and LD.V treatments values of thermic quanta (graph T) for all the varieties are low and the corresponding values of photo quanta (graph P) are high. Values of thermic quanta (graph T) are higher for ND.C and ND.V treatments and therefore the corresponding values of photo quanta (graph P) are lower. This inverse relationship is further borne out by a further rise in the thermic quanta (graph T) and a corresponding fall in the photo quanta of SD.C and SD.V treatments. Under both SD and ND treatments the relative positions of graphs P and T are reversed compared with LD treatment. The fact that this inverse relationship between the light and temperature requirements of a variety is not fortuitous has been abundantly proved by growing plants of the same variety in differ-

ent ranges of temperatures. Photothermic and vernalization quanta of the twelve varieties progressively increase with the length of their vegetative periods (Table 3).

General Discussion

This discussion will be confined to the quantitative relationship between light and temperature as affecting the vegetative period of a plant.

Dolgusin and Lysenko (1929) have attempted to obtain a quantitative relationship between the temperature and the length of the thermo phase, and have tried to express this relation by a formula. This relationship is, however, confined to the thermo phase of the plant only. These workers have also made it quite clear that the durations of the photo phase and the subsequent phases are inversely related to temperature. Kopetz (1943) has developed the concept of 'pure warmth sum', which according to him, is a constant for a species. The thermic quantum (T) as suggested in this paper is comparable to the 'pure warmth sum' of Kopetz, and as can be seen from the results (Figures 2 and 4) is not a constant for any variety of wheat under different conditions of light and temperature. 'The least variable numerical expression' determined by Nuttonson (1948) as the product of average day length and summation of day degrees of the vegetative stage from the phenological data of a number of varieties of crop plants is not based upon a study of the vernalization and photoperiodic responses of a plant; and also it does not take into consideration the effect of temperature on the duration of the thermo phase.

The concept of photothermic quantum as well as vernalization quantum suggested in this paper is based upon the vernalization and the photoperiodic responses of wheat varieties and differs from the least variable numerical expression of Nuttonson in that, that the total number of light hours during the vegetative period are used to derive the product index and not the mean day length. The advantages of these indices over those of the prvious workers will become obvious from the discussion that follows.

Vernalization quantum is an index of the energy requirement of a wheat variety for the completion of its thermo phase. It increases with the length of the vegetative period of a variety and is the largest for the winter varieties. Space does not permit a fuller discussion of the concept of vernalization quantum in relation to Lysenko's concept of thermo phase. However, in Experiment III photothermic and vernalization quanta of well known winter varieties of wheat like, Triticum vavilovianum, var. Vaneum Yakub, T. turgidum, variety Cambridge Rivett, and T. vulgare, var. Yeoman II have been determined. The results clearly demonstrate that the unvernalized plants of

Table 4. *Photothermic quanta of three winter varieties under the three light treatments as well as for unvernalized and vernalized plants.*

Variety	Unvernalized			Vernalized		
	(LD)	(ND)	(SD)	(LD)	(ND)	(SD)
Yeoman II	18.30	16.83	16.82	15.03	15.27	14.99
Vaneum Yakub	19.25	17.49	16.99	16.03	14.90	14.57
Cambridge Rivett	19.79	18.45	18.09	14.64	14.86	15.00

Multiply the values in the Table by 10^6 to obtain the values of photothermic quanta.

these winter types give higher values for photothermic quanta under continuous light as compared to unvernalized plants of these varieties under normal and short day treatments. Photothermic quanta of the above mentioned three winter varieties for the three light treatments are presented in Table 4. Vernalized plants of these varieties do not show such differences. This fact clearly indicates that unless the thermo phase is quickly completed as suggested by Lysenko a part of the light energy will not be efficiently utilized by the plant.

The quantitative relationship between the photothermic quantum, the vernalization quantum, and the length of the vegetative period of wheat has an important bearing upon physio-ecological and physio-genetical problems. The photothermic quantum (E) of a variety is the product of the photo (P) quantum of the variety and its thermic (T) quantum.

The photo quantum of a variety in its turn is a product of the mean diurnal photoperiod (p), and the vegetative period (F) of the variety; while the thermic quantum is the product of the mean daily temperature (t) and the vegetative period of the variety. It, therefore, follows that: —

$$E = PT = ptF^2, \text{ and } \therefore F = \sqrt{\frac{E}{pt}}$$

It would appear from the foregoing that if the photothermic quantum of a variety is known, the approximate length of the vegetative period of a variety can be worked out for any given environment from the above formula by knowing the mean daily temperature and the mean day length during its growing season. The vernalization quantum of a variety will have to be taken into account also. If the temperature at the beginning of the growing season is conducive to the rapid completion of the thermo phase the vernalization quantum will be small and the photothermic quanta for control and vernalized plants will not materially vary because the light energy will be efficiently utilized by the plant for the completion of the developmental process and consequently the vegetative period will need no

correction. If, on the other hand, a variety is grown in a range of temperature which retards the processses of the thermo phase (e.g. by growing winter varieties in a warm climate) the vegetative period will be prolonged owing to the inefficient utilization of light energy by the unvernalized plant, and therefore a correction of its vegetative period will be necessary.

A reference has already been made to the work of Went and his collaborators (1944—1948) on thermoperiodicity. The data presented by Lewis and Went (1945) for thirteen species of California annuals under twelve different controlled conditions of temperature and photoperiod in air conditioned greenhouses were examined in the light of the concept of the photothermic quantum elaborated in this paper. Paucity of space precludes a fuller presentation of this analysis here. However, it will not be out of place to mention that calculations of photothermic quanta for these species from the data of vegetative periods, photoperiods and temperatures given by these workers, on the basis of the above mentioned formula, showed that these values of photothermic quanta for the plants of any given species agreed fairly well even though they were placed under widely differing controlled environments. Although in majority of species values of photothermic quanta agreed fairly well in some cases there were wide divergencies in these values probably on account of the very considerable differences in the growing conditions. It may also be noted that the data given by Lewis and Went are not adequate for such calculations; and also that two of the species were short day plants. No account has also been taken by these workers to determine the environmental requirements of these species for the completion of their thermo phase. As already shown above the vernalization quantum of a plant appreciably influences its photothermic quantum.

A mention has already been made of the differential photosesitivity of wheat varieties previously (Chinoy, 1950) as well as in this paper. Photothermic quanta required for the flowering of different varieties belonging to different flowering classes show that lesser energy quantum is necessary for flowering of early varieties compared to late ones. This suggests that an early variety has a greater sensitivity to photothermic energy. which enables it to complete the developmental process with lesser amount of energy obtained from the environment; whereas the lesser photosensitivity of a late flowering variety necessitates the utilization of a greater amount of photothermic energy for the completion of the same process. Early or late flowering of a variety in a given environment will, therefore, be determined by the time needed to meet its energy requirement.

The concept of the photothermic quantum has also been applied to segregating populations derived from a number of wheat and linseed crosses grown under different photoperiodic and vernalization treatments, as well

as to other crop plants like, barley, oats, rye, gram, linseed, etc. While leaving a fuller discussion of this mass of data to subsequent communications it may be pointed out that the concept of photothermic quantum for the so-called long day plants is a useful and a workable index of energy requirement of a plant.

In conclusion an interesting fact may be recorded here. The photothermic quantum of a variety under different environmental conditions in a given season varies only within a comparatively narrow range of experimental error; whereas on the other hand, variations in the photothermic quantum of a variety determined in different seasons are appreciably greater, and in some varieties (especially winter varieties) regular trends in these variations are discernible (unpublished records). To some extent these variations may be ascribed to differences in environmental conditions which are not detected and therefore not taken into account in determining the photothermic quanta. However, regular trends of variations in the photothermic quanta of a variety, determined year after year, cannot be fully explained on the above basis alone. It appears as if the environmental influences are leaving their impress upon each succeeding generation of plants in a regular and cumulative manner.

Summary

1. In three complex experiments involving different treatment combinations, unvernalized and vernalized plants of a number of Indian and exotic varieties of wheat were grown under a number of photoperiodic treatments comprising of varying numbers of Long (24-hour) Days, and Short (6 or 7-hour) Days, as well as Normal Days of 11 hours of illumination.

2. Total number of light hours (Photo Quantum — P) as well as the sum of degrees of mean diurnal temperatures (Thermic Quantum — T) of Vegetative Periods (number of days from the date of germination or transplantation to the date of anthesis) were calculated for plants under different treatment combinations. Regression of the logarithm of the photo quantum on the logarithm of the thermic quantum of a variety was determined for unvernalized and vernalized plants of each variety individually.

3. Products of photo and thermic quanta of all varieties under different light and vernalization treatments were worked out and were found to agree closely for a variety under different treatments. This product is named the *Photothermic Quantum* of a variety.

4. The difference between the photothermic quanta of unvernalized and

vernalized plants of a variety has been designated as its *Vernalization Quantum.*

5. Photothermic and vernalization quanta of wheat varieties increased progressively with lengths of their vegetative periods. The relation between the photothermic quantum (E) of a variety, the length of its vegetative period (F), the mean temperature (t) during the vegetative period, and the mean photo period (p) is expressed by the following equation: $E = PT = ptF^2$; and $\therefore F = \sqrt{\dfrac{E}{pt}}$.

6. If the vernalization quantum of a variety is large it should be taken into account in calculating the vegetative period by the above formula.

7. Results of other workers have been discussed in the light of the concepts of photothermic and vernalization quanta as elaborated in this paper. It is suggested that the photothermic and the vernalization quanta can serve as useful and workable indices of energy requirements of long day plants.

References

1. Bakhteyev, F. Kh.: (Geographical variation of the length of the vegetation period in various barley types). — Publication of the All-Union Lenin Academy of Science, Moscow, 1935.

2. Borthwick, H. A., Parker, M. W. & Heinze, P. H.: Effect of Photoperiod and temperature on development of barley. — Bot. Gaz., 103: 326, 1941—42.

3. — — & Scully, N. J.: Effects of Photoperiod and temperature on growth and development of Kok-Saghyz. — Ibid. 105: 100. 1943.

4. Chinoy, J. J.: Correlation between growth and development. — Nature (London) 164: 879. 1949.

5. — Effect of vernalization and photoperiodic treatments on grain development in wheat. — Curr. Sci. 18: 414. 1949 a.

6. — Effect of vernalization and photoperiodic treatments on growth and development of wheat. — Nature (London) 165: 882. 1950.

7. — & Nanda, K. K.: Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part I. Varietal differences in flowering of wheat and its correlation with spike growth, under varying photoinductive and post-photoinductive treatments. — Physiol. Plant. 4: 209. 1951 a.

8. — — Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part II. Varietal differences in stem elongation and tillering of wheat and their correlation with flowering under varying photoinductive and post-photoinductive treatments. — Ibid. 4: 427. 1951 b.

9. — — Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part III. Rate of dry matter production, net assimilation rate and water content of wheat under varying photoinductive and post-photoinductive treatments. — Ibid. 4: 575. 1951 c.

10. — — Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part IV. Uptake of nitrogen, phosphorus, and potassium

by wheat plant under varying photoinductive and post-photoinductive treatments. — Ibid. 5: 11. 1952.

11. Dolgŭsin, D. A. & Lysenko, T. D. — Trudy Vsesojuz. S'esda Genet. Selek. Semenovod. plemennomu Zivot. 3: 189. (see Whyte, 1949).

12. Garner, W. W. & Allard, H. A.: Effect of the relative length of day and night and other factors of environment on growth and reproduction in plants. — J. Agri. Res. 18: 553. 1920.

13. Gassner, G.: Beiträge zur physiologischen Charakteristik sommer- und winterannueller Gewächse, insbesondere der Getreidepflanzen. — Zeits. für Bot. 10: 417. 1918.

14. Hamner, K. C.: Pflanzenbau. — 15: 1. 1938. (Whyte, 1949).

15. — Photoperiodism in Plant. — Ann. Rev. Biochem. 13: 575. 1944.

16. Klebs, G.: Über die Blutenbildung von Sempervivum. — Flora 111: 128. 1918.

17. Kopetz, L. M.: — Gartenbauwiss. 17: 255. 1943. (Whyte, 1949).

18. Lang, A. & Melchers, G.: Die photoperiodische Reaktion von Hyoscyamus niger. — Planta 33: 651. 1943.

19. Lewis, H. & Went, F. W.: Plant growth under controlled conditions. IV. Response of California annuals to photoperiod and temperature. — Amer. J. Bot. 32: 1. 1945.

20. Lysenko, T. D.: Theoretical Basis of Vernalization. — Moscow, 1935.

21. Naylor, A. W.: Effects of some environmental factors on photoperiodic induction of beet and dill. — Bot. Gaz. 102: 557. 1941.

22. Nuttonson, M. Y.: Some preliminary observations of phenological data as a tool in the study of photoperiodic and thermal requirements of various plant material. — Vernalization and Photoperiodism (A Symposium),; Chronica Botanica Co. Waltham, Mass., U.S.A. pp. 129. 1948.

23. Parker, M. W. & Borthwick, H. A.: Influence of temperature on photoperiodic reactions. — Bot. Gaz. 104: 612. 1943.

24. Purvis, O. N.: An analysis of the influence of temperature during germination, on the subsequent development of certain winter cereals and its relation to the effect of length of day. — Ann. Bot. 48: 919. 1934.

25. — & Gregory, F. G.: Studies in vernalization of cereals. I. A comparative study of vernalization of winter rye by low temperature and by short days. — Ann. Bot. N. S. 1: 569. 1937.

26. Rudorf, W. & Schroeck, O.: Z. Pflanzenz. 24: 108. 1941. (Whyte, 1949).

27. Sakr, El Sayd: Proc. Amer. Soc. hort. Sci. — 44: 473. 1944. (Whyte, 1949).

28. Thompson, H. C.: Proc. Amer. Soc. hort. Sci. 37: 672. 1939. (Whyte, 1949).

29. Voss, J.: Pflanzenbau. 15: 1. 1938. (Whyte, 1949).

30. Went, F. W.: Plant growth under controlled conditions. II. Thermoperiodicity in growth and fruiting of the tomato. — Amer. J. Bot. 31: 135. 1944.

31. — Plant growth under controlled conditions. III. Correlation between various physiological processes and growth in the tomato plant. — Amer. J. Bot. 31: 597. 1944.

32. — Simulation of photoperiodicity by thermoperiodicity. — Science. New Series 101: 97. 1945.

33. — Thermoperidiocity. Vernalization and Photoperiodism (A Symposium). Chronica Botanica Co., pp. 145. 1948.

34. Whyte, R. O.: Crop Production and Environment. — Faber and Faber Ltd. London, 1949.

The Assimilation of Ammonia and Nitrate by Nitrogen-Starved Cells of Chlorella vulgaris

II. The Assimilation of Large Quantities of Nitrogen

By

P. J. SYRETT

Botany Department, University College, London
(Received September 29, 1955)

Introduction

Nitrogen-starved cells of *C. vulgaris* assimilate added nitrate or ammonium-N rapidly and the assimilation is accompanied by marked changes of respiration rate. When small quantities of nitrate or ammonium-N are added assimilation continues until all the added nitrogen has been assimilated; the respiration rate then returns to the control value (Syrett, 1955). When a large quantity of ammonium-N is added rapid assimilation continues until the available carbohydrate inside the cells is exhausted (Syrett, 1953 a). In this and the following papers the metabolism which accompanies the assimilation of large quantities of ammonium-N and nitrate-N is studied. Sufficient nitrate or ammonium-N was added to nitrogen-starved cells so that some still remained in the medium when rapid assimilation had ceased. An increased rate of respiration and a decrease of intracellular carbohydrate accompanied the assimilation of both forms of nitrogen but there were interesting differences between the nitrate-treated and ammonium-treated cells.

Experimental

Material. — *Chlorella vulgaris* (Pearsall's strain) was grown in pure cultures as described previously (Syrett, 1953). The nitrogen in the growth medium was supplied as ammonium nitrate. The medium was buffered at pH 6.1 with 0.067 M phosphate.

P. J. SYRETT

The cultures were grown at 25° C and illuminated by a tungsten filament lamp giving a light intensity of 600 f.c. at the culture surface. They were aerated by a stream of sterile air containing 0.5 per cent carbon dioxide and flowing at the rate of 10 l./hr. through each culture vessel.

After three days growth, cultures were harvested by centrifuging at 360 g. for 5 mins. The cells were washed and resuspended in sterile nitrogen-free medium (the growth medium with ammonium nitrate omitted). These cultures were then maintained for a further 16—20 hrs. under the growth conditions described above but with an increased gas flow of 20 l./hr. per culture. Immediately before starting the nitrogen assimilation experiments, the cells were centrifuged, washed and resuspended in 0.067 M phosphate with 0.0017 M magnesium sulphate, pH 6.1—6.2. The final suspension contained about 8 mgs. dry wt. cells/ml.

The ammonium sulphate and potassium nitrate used were also dissolved in the suspending medium.

Assimilation and respiration experiments. Several experiments were made; these were all fundamentally the same in design but differed in detail. Known volumes of the cell suspension were pipetted into conical flasks which were shaken in a darkened tank at 25° C. At a known time ammonium sulphate, potassium nitrate or nitrogen-free buffer was added to the flasks. Since the rate of nitrate assimilation was slower, usually twice as much ammonium-N was added as nitrate-N. This increased the accuracy of the nitrate estimations. Preliminary experiments showed that the concentration of ammonium-N or nitrate-N had no effect on the rate of assimilation over the range used.

Aliquots for the determination of nitrogen fractions, carbohydrates etc. were removed from the flasks at intervals and centrifuged at once. The supernatant was decanted from the cells and the centrifuge tube containing the cells was immersed in a boiling water bath for 3 mins. The further treatment of the cells is described below.

2.0 ml. samples of the same original suspension were pipetted into Warburg flasks for determinations of respiration. Ammonium sulphate, potassium nitrate or nitrogen-free buffer was added from the side arm. Thus the respiration determinations were made concurrently with the assimilation experiments. Analysis of the residual ammonium-N and nitrate-N in the Warburg flasks at the end of the experiments showed that this was the same as in the large assimilation flasks so it is concluded that the conditions in the Warburg vessels did not differ significantly from those in the assimilation flasks.

Analytical methods

Ammonium and nitrate-N. — In experiment I ammonium-N and nitrate-N were determined by pipetting 0.25 ml. of cell suspension into a Conway unit which contained Devarda's alloy when nitrate was to be determined (the cultures with added nitrate were shown to be free of nitrite and ammonia). Ammonia was released by the addition of alkali (Conway, 1947) and absorbed in 0.05 N sulphuric acid in the central chamber of the unit. After 2—3 hrs. the contents of the central chamber were transferred to a graduated tube and ammonia determined colorimetrically with Nessler reagent (Folin & Denis, 1916). The light absorption was measured at 450 mμ. Freshly prepared standards were measured at the same time.

In experiment II ammonium-N and nitrate-N were determined on 0.5 ml. samples of the supernatant after removal of the cells by centrifuging. Previous work had shown that the ammonia and nitrate in the cells was negligible. Ammonium-N was determined by Conway's method as outlined above. Nitrate-N was determined colormetrically with phenolsulphonic acid, the colour produced being read at 430 mμ. Standards were treated similarly at the same time.

Other nitrogen fractions. — The killed cells in the centrifuge tube were freeze-dried until they could be extracted. They were then extracted with three successive portions of water at 80° C. The combined extracts were made up to a definite volume and aliquots taken for the determination of the various fractions. In experiment I the residue, after extraction, was digested by the Kjeldahl method, the digest made up to volume and the ammonia in aliquots determined as outlined above. In this way the insoluble-N of samples was determined directly.

The following fractions were determined in the extracts (for further details see Syrett 1953 b). All analyses were in duplicate or triplicate.

Soluble organic-N. — This was determined as ammonia after Kjeldahl digestion followed by a Conway distillation. Extracts from the nitrate treated cells were reduced with reduced iron before digestion. A correction for free ammonium-N or nitrate-N in the extracts was made.

Amide-N. — 0.5 ml. extract was heated for 3 hrs. at 100° C with 0.25 ml. 3 N sulphuric acid. The ammonia produced was determined aften a Conway distillation. A correction for free ammonia present was made.

α-amino-N. — The colorimetric method of Moore & Stein (1948) was used after the removal of ammonia.

Carbohydrate determinations. — The killed cells in the centrifuge tubes were freeze-dried until they could be extracted. Soluble carbohydrates were extracted with 70 per cent ethanol (three extractions, each for 24 hrs. at 25° C were made). The extract was dried at 80° C and the residue taken up in water. The solution was cleared with lead acetate followed by phosphate. The total soluble carbohydrate in this extract was determined by the Hagedorn-Jensen method after acid hydrolysis (0.4 N sulphuric acid for 10 mins. at 100° C).

The insoluble cell residue remaining after extraction was hydrolysed for 3 hrs. at 100° C with 3 per cent v/v sulphuric acid. After neutralising, the reducing power of this solution was determined and taken as insoluble polysaccharide. The value determined was increased by 3 per cent to allow for losses on hydrolysis.

The sum of the soluble and insoluble polysaccharide is referred to as total carbohydrate.

Total ether-soluble material. — This was determined on samples taken from experiment II only. 4 ml. aliquots of suspension were centrifuged, the supernatant discarded and the cells killed by immersing the centrifuge tube in a boiling water bath for 3 mins. 2 ml. 95 per cent methanol (redistilled) was then added to the cells and they were left until the extraction could be completed. To do this, the cells were resuspended in the methanol and the tube heated to 70° C for 15 mins. The tube was then centrifuged and the extract decanted. Two further extractions with methanol were then carried out and these left a pale-yellow insoluble residue. This residue was extracted with 3 portions of 2 ml. ether. The extractions were made at 35° C for about 15 mins. The last extract was quite colourless and the cell debris was also colourless at the end.

The methanol extracts were mixed with ether and water added so that a dark

green ether layer separated. This ether layer was removed and two further extractions of the methanol with ether were carried out. These ether extracts were combined and added to those of the cell debris. The whole ether extract from any one sample was placed in a weighed tube and dried to constant weight at 50° C in a slow stream of nitrogen. The weight of the ether soluble material was determined to 0.01 mg. on a semi-micro balance. A preliminary determination on three replicate samples by this method gave values which differed from the mean by less than 2 per cent; in these samples the total ether soluble material was 21 per cent of the dry weight.

Respiration measurements. — Each Warburg flask contained 2.0 ml. cell suspension and the appropriate volume of ammonium sulphate, potassium nitrate or nitrogen-free buffer was added from the side arm. Oxygen uptake was determined from flasks with caustic potash in the centre well. Carbon dioxide production was calculated by the Warburg direct method (Dixon 1949) and retention allowed for in two ways. Firstly the pH of the medium was accurately determined and the correction of Umbreit, Burris and Stauffer (1949) applied, and secondly, 0.2 ml. 10 N sulphuric acid was added from a second side-arm at the end of the experiment so that the final volume of carbon dioxide produced could be accurately determined. The two methods gave virtually the same result when applied to the control flasks but there is a decrease of pH during ammonium-N assimilation and an increase during nitrate assimilation; consequently the two methods gave different values for carbon dioxide production in the experimental flasks. A combination of both methods was used, therefore, to calculate the probable true course of carbon dioxide production assuming that the correction of Umbreit *et al* is approximately correct just after the addition of ammonium or nitrate-N when the pH has changed little and that the correct final volume of carbon dioxide is given after the addition of acid at the end of the experiment.

Experimental Results and Discussion

Experiments I and II were similar; the results are shown in Figures 1 and 2. Only the changes of nitrogen fractions and ether soluble material are plotted for experiment II; the changes of respiration and carbohydrates were similar to those of experiment I.

Nitrogen fractions. — The experiments show that nitrogen-starved cells assimilate ammonium-N 4—5 times more quickly than nitrate-N. The rate of ammonium assimilation falls continuously from the beginning of the experiment while that of nitrate assimilation remains constant for some 80 minutes. After 100 minutes the assimilation of both forms of nitrogen has practically ceased and, at this point, the cells supplied with ammonium-N (ammonium cells) have assimilated 3—4 times as much nitrogen as the nitrate cells.

Much of the nitrogen assimilated by the ammonium cells remains as soluble organic nitrogen. This fraction increases rapidly and is more than
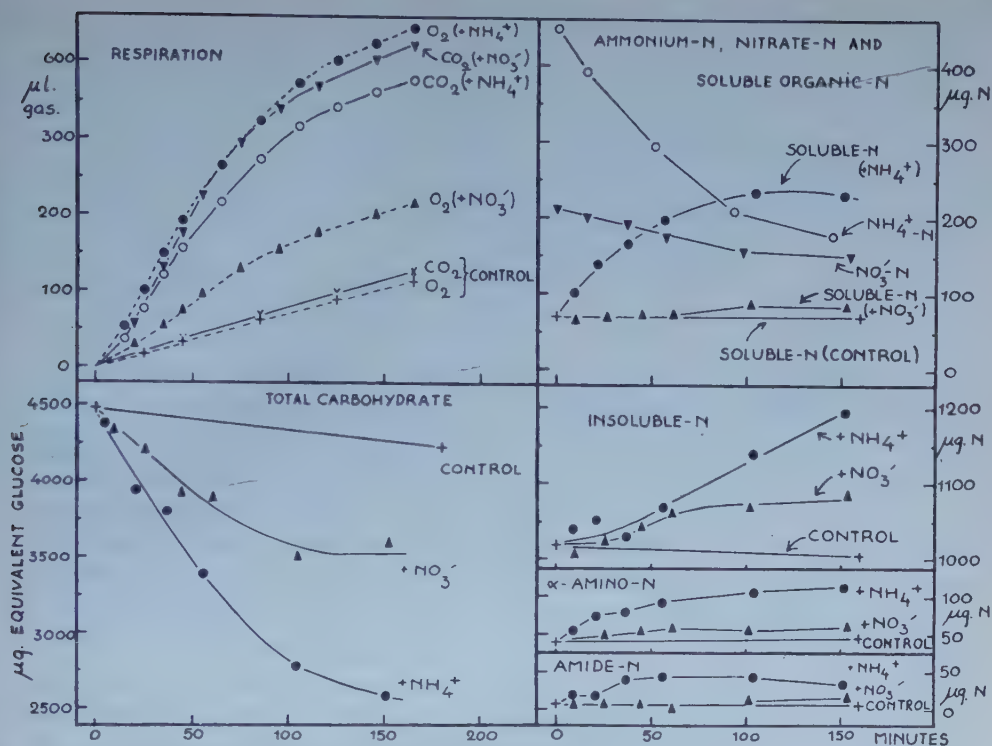
Figure 1. *Experiment I. The changes in gas exchange, total carbohydrate, and nitrogen fractions following the addition of ammonium-N and nitrate-N to nitrogen-starved Chlorella. The results are given as μg. or μl. per 2.0 ml. of original cell suspension (2.2 ml. after additions); this contained 13.6 mg. dry wt. cells. Initial nitrogen concentration 15 μ-atoms ammonium-N or 7.5 μ-atoms nitrate-N per ml. Initial pH 6.15; 0.0067 M phosphate.* -O—O-, -●—●- ,ammonium-N added; -▲—▲-, -▼—▼-, Nitrate-N added; -×—×-, -+—+-, control.

twice its initial value when assimilation ceases. The increase is largely due to the synthesis of amino acids and amides; the basic amino acids are particularly important (Syrett and Fowden, 1952). The soluble-N fractions of the nitrate cells, however, increase little.

Insoluble-N, presumably chiefly protein, is synthesised more rapidly from ammonium-N than from nitrate but the difference in the rates of increase of this fraction is not nearly so marked as it is for soluble organic-N. When ammonium-N is assimilated the rate of synthesis of amino-acids is much greater than the rate of their conversion to protein but when nitrate is assimilated, the two rates must be more nearly equal. A similar difference was observed by Virtanen, Czaky and Rautenen (1949) when nitrogen deficient Torula assimilated ammonium or nitrate-N but in their experiments the rate of protein synthesis was the same with either nitrogen source.
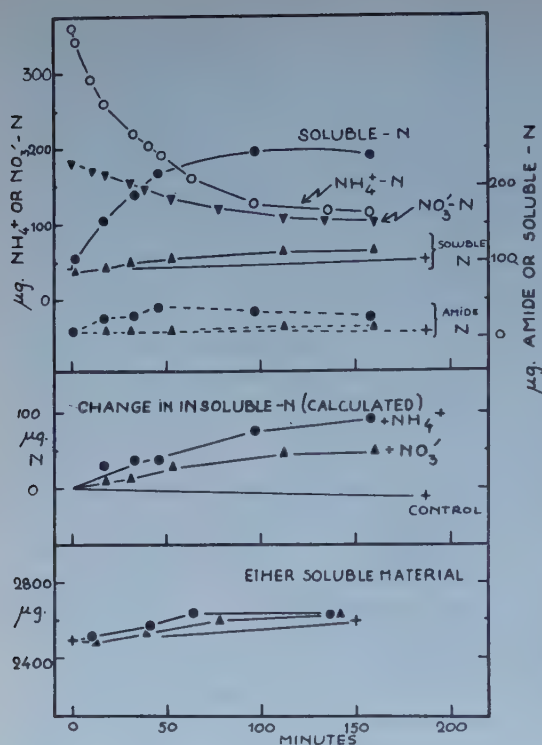
Figure 2. *Experiment II. The change in nitrogen fractions and ether soluble material following the addition of ammonium-N and nitrate-N to nitrogen-starved* Chlorella. *The results are given as μg. per 2.0 ml. of original suspension (2.2 ml. after additions); this contained 16.4 mg. dry wt. cells. Initial nitrogen concentration 12 μ-atoms ammonium-N or 6 μ-atoms nitrate-N per ml. Initial pH 6.16* -○—○-, -●—●-, *ammonium-N added;* -▲—▲-, -▼—▼-, *nitrate-N added;* -×—×-, -+—+-, *control.*

In experiment I, insoluble-N was determined on the residue remaining after the extraction of soluble-N; the scatter of the earlier points is a reflection of the difficulty of determining accurately changes of the order of 2—3 per cent. However, the determined values can be compared with the increase calculated from the difference between the amount of ammonium-N or nitrate-N which has disappeared and the increase of soluble organic-N. The observed and calculated values agree well — the determined values being somewhat higher. The agreement indicates that no nitrogen is lost from the cultures during the experimental period.

Gas exchange. — An immediate increase of respiration rate follows the addition of either nitrate or ammonium-N to nitrogen starved cells. The rates of gas exchange immediately after the additions are shown in Table 1. The addition of ammonium-N is followed by a large increase of the rates of both oxygen uptake and carbon dioxide production but oxygen uptake increases more so that the R.Q. during this period is about 0.8. This is presumably associated with the conversion of carbohydrate to more oxidised organic-N compounds. After nitrate addition the rate of oxygen uptake doubles but

Table 1. *Gas exchange during period 10—20 minutes after the addition of ammonium-N or nitrate-N to nitrogen-starved Chlorella.* (µl. per 2.2 ml. suspension.)

Addition	Oxygen		Carbon dioxide		R. Q.	
	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
N-free buffer	— 7	— 6	8	7	1.15	1.16
Nitrate-N.........................	— 17	— 14	40	40	2.35	2.85
Ammonium-N	— 51	— 50	42	39	0.83	0.78

For experimental details, see legends to Figures 1 and 2.

the rate of carbon dioxide production increases much more so that the R.Q. is high. It is interesting that, at this period, the rates of carbon dioxide production are the same with either nitrogen compound although later the rate of production by the ammonium cells drops so that finally the nitrate cells produce rather more carbon dioxide (Figure 1).

The assimilation of ammonium-N ceases when the available carbohydrate is exhausted (Syrett 1953 a). Figure 1 shows that nitrate-N assimilation stops at about the same time as ammonium-N assimilation and that the time-curves for carbon dioxide production are similar. In an earlier paper (Syrett, 1954), it was concluded from this that the available carbohydrate was used up at about the same rate in both ammonium and nitrate cells. Carbohydrate analyses, however, show that this conclusion is erroneous. Carbohydrate is used up more quickly in the ammonium cells and, when assimilation stops, the nitrate cells contain appreciably more carbohydrate than the ammonium ones. This point will be considered more fully in the following paper.

Ether-soluble material. — Nitrogen deficient algae are known to accumulate lipides (Fogg and Collyer, 1953) and it was possible that the amounts of these change rapidly during nitrate or ammonium-N assimilation. Figure 2 shows, however, that this is not so. The change is slight, a small increase occurring during the experiment; this may be due to chlorophyll synthesis which is known to accompany the assimilation of nitrogen by nitrogen starved *Chlorella* (Van Hille, 1938). Chlorophyll may account for as much as 30 per cent of the ether soluble material of *Chlorella* (Milner 1953).

It is clear that the contribution of lipide to the carbon available for the synthesis of organic-N compounds is negligible. Possibly lipides are more important in algae such as *Chlorella pyrenoidosa* which, when nitrogen deficient, accumulates much larger quantities than *C. vulgaris*. (Fogg and Collyer). Millbank (1954) has shown that the amount of organic acid in nitrogen-starved Chlorella is small (less than 1 µg. per mg. dry wt.). Consequently it must be chiefly carbohydrate carbon which is available for respiration and the synthesis of organic nitrogen compounds.

Table 2. *Changes in cell number during Experiment II*. Number of cells/cu. mm. ($\times 10^{-3}$.) The final samples were taken 160 mins. after the addition of the solutions. For experimental details see legend to Figure 2.

Addition	Initial count	Final count
N-free buffer	338 ± 9.8	328 ± 9.6
Nitrate-N	293 ± 9.1	334 ± 9.7
Ammonium-N	296 ± 7.4	321 ± 9.5

Cell growth during assimilation. — Protein is presumably synthesised during the assimilation experiments since the insoluble-N in the cultures increases. No appreciable cell division, however, takes place (Table 2).

The results presented here will discussed more fully in the following paper after further experiments have been described.

Summary

Nitrogen-starved cells of *C. vulgaris* assimilate ammonium-N about 4 times as quickly as nitrate-N at pH 6.1. The assimilation of both forms of nitrogen is accompanied by a high respiration rate and a decrease of intracellular carbohydrate. Carbohydrate disappears more rapidly from the ammonium-treated cells.

When nitrate-N is assimilated soluble organic nitrogen compounds increase little but in the ammonium cells they treble in amount. Insoluble-N is synthesised twice as quickly in the ammonium cells.

The rate of carbon dioxide production accompanying the assimilation of both forms of nitrogen is the same and 5 times higher than the control rate. The assimilation of ammonium-N is accompanied by a high rate of oxygen uptake and the R.Q. is about 0.8; the rate of oxygen uptake accompanying nitrate assimilation is lower and the R.Q. about 2.5.

Most of the carbon available for synthesis and respiration is carbohydrate carbon. Ether soluble material increases slightly during the period of nitrogen assimilation.

The assimilation of both forms of nitrogen ceases after the same period of time and, when this happens, the cultures still contain inorganic nitrogen. When assimilation ceases the ammonium treated cells contain considerably less carbohydrate than the nitrate cells.

References

Conway, E. J.: Microdiffusion analysis and volumetric error. — London: Crosby Lock-wood & Son, Ltd. 1947.

Dixon, M.: Manometric methods. — Cambridge University Press. 1949.

Fogg, G. E. & Collyer, D. M.: The accumulation of lipides by algae. — In Algal culture, Carnegie Institution of Washington publication No. 600: 177. 1953.

Folin, O. & Denis, W.: Nitrogen determinations by direct Nesslerisation. — J. Biol. Chem. 26: 473. 1916.

van Hille, J. C.: The quantitative relation between rate of photosynthesis and chlorophyll content in Chlorella pyrenoidosa. — Rec. Trav. Bot. Néer. 35: 680. 1938.

Millbank, J. W.: A study of intermediary metabolism in two micro-organisms with parti-cular reference to keto-acids. — Ph. D. thesis University of London. 1954.

Milner, H. W.: The chemical composition of algae. — In Algal Culture, Carnegie Institution of Washington publication No. 600: 285. 1953.

Moore, S. & Stein, W. H.: Photometric ninhydrin method for use in the chromatography of amino-acids. — J. Biol. Chem. 176: 367. 1948.

Syrett, P. J.: The assimilation of ammonia by nitrogen-starved cells of Chlorella vul-garis. Part I. The correlation of assimilation with respiration. — Ann. Bot. Lond. N.S. 17: 1. 1953 a.

— Part II. The assimilation of ammonia to other compounds. — Ann. Bot. Lond. N.S. 17: 21. 1953 b.

— Ammonia and nitrate assimilation by green algae (Chlorophyceae). — In Auto-tropic organisms. Symposium of the Society for General Microbiology. 1954.

— The assimilation of ammonia and nitrate by nitrogen-starved cells of Chlorella vulgaris. I. The assimilation of small quantities of nitrogen. — Physiol. Plant. 8: 924. 1955.

— & Fowden, L.: The assimilation of ammonia by nitrogen-starved cells of Chlorella vulgaris. III. The effect of the addition of glucose on the products of assimilation. — Physiol. Plant. 5: 558. 1952.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: Manometric techniques and tissue meta-bolism. — Minneapolis: Burgess Publishing Co. 1949.

Virtanen, A. I., Csáky, T. Z. & Rautanen, N.: On the formation of amino-acids and proteins in Torula utilis on nitrate nutrition. — Biochim. et Biophys. Acta, 3: 208. 1949.

The Assimilation of Ammonia and Nitrate by Nitrogen-Starved Cells of Chlorella vulgaris

III. Differences of Metabolism Dependent on the Nature of the Nitrogen Source

By

P. J. SYRETT

Botany Department, University College, London
(Received September 29, 1955)

Introduction

In the previous paper (Syrett, 1956) it was shown that the addition of either ammonium or nitrate-N to nitrogen-starved cells of Chlorella is followed by the rapid assimilation of the added nitrogen accompanied by a rapid carbon metabolism. In the experiments described in that paper, nitrogen assimilation continued rapidly for 70—100 minutes and then stopped, although the cultures still contained ample ammonium or nitrate-N; rapid assimilation continued for about the same time whichever nitrogen source was added. However, when assimilation ceased the nitrate cells contained considerably more intracellular polysaccharide than the ammonium cells. Earlier work (Syrett, 1953 a) suggested that ammonia assimilation ceases when the cells have exhausted their intracellular polysaccharide but, if this so, it is not clear why similar cells should cease to assimilate nitrate while they still contain much intracellular polysaccharide. The experiments described in this paper were carried out to investigate this further.

Methods

The methods by which the cells were grown and starved of nitrogen are described in the previous paper, as are most of the analytical methods. Sampling experiments

[28]

were carried out on cultures shaken in large conical flasks in darkness, at the same time as respiration experiments on portions of the same suspension. The technique is fully described in the previous paper.

Chromatograms of sugars produced from insoluble polysaccharide by hydrolysis. — A salt-free solution of the sugars resulting from the hydrolysis of insoluble polysaccharide was obtained by neutralising the hydrolysate with barium hydroxide. Chromatograms of these solutions were run on Whatman No 4 paper in the ethyl acetate-acetic acid solution of Jermyn and Isherwood (1949). After drying the paper was sprayed with the aniline-phthalate spray of Partridge (1949).

Determination of glucose-polysaccharide. — The chromatograms showed that the only important sugars in the extracts were glucose and mannose. The glucose was destroyed by incubation of aliquots of the hydrolysate with glucose oxidase (from Sigma Chemical Co.) at 37° C for 3 hrs. After incubation the reducing value of the mixture was determined and compared with that of a similar sample which had not been incubated with the enzyme. The difference is taken to be equal to the reducing value of the glucose in the sample. A correction was applied for the reducing value of the enzyme solution. Preliminary work showed that this method destroyed 97 per cent of the glucose and only 2—3 per cent of the mannose in mixtures of the two sugars. The method is described more fully by Keilin and Hartree (1948).

Experimental

It was possible that rapid nitrate assimilation ceased after 70—100 minutes in the earlier experiments because the cells had recovered from nitrogen starvation and reverted to the slower rate of assimilation of normal non-nitrogen starved cells.

This possibility was investigated in two ways. Firstly nitrate was supplied to nitrogen-starved cells with and without glucose. When glucose was added, the cells assimilated nitrate at a rate which remained constant until all the nitrate had been assimilated (160 minutes) (Figure 1). The cells without added glucose, ceased to assimilate nitrate after 70 minutes. The rate of assimilation of the cells with glucose was about twice as rapid (on a dry wt. basis) as that of normal cells with glucose. Thus, providing glucose is present, the nitrogen-starved cells can continue to assimilate nitrate and there is no evidence of recovery from nitrogen starvation during the experimental period.

Figures 1 and 2 illustrate the second approach to this problem. Cells were allowed to assimilate nitrate, without added glucose, until assimilation ceased. At this point their carbohydrate content was higher than that of similar cells which had assimilated ammonium-N. Ammonium-N was then added to the nitrate cells and was immediately assimilated rapidly. The assimilation was accompanied by an increased respiration rate and the carbohydrate content of the cells fell to the level of the ammonium cells. The change in soluble carbohydrate is particularly interesting. This fell quickly when
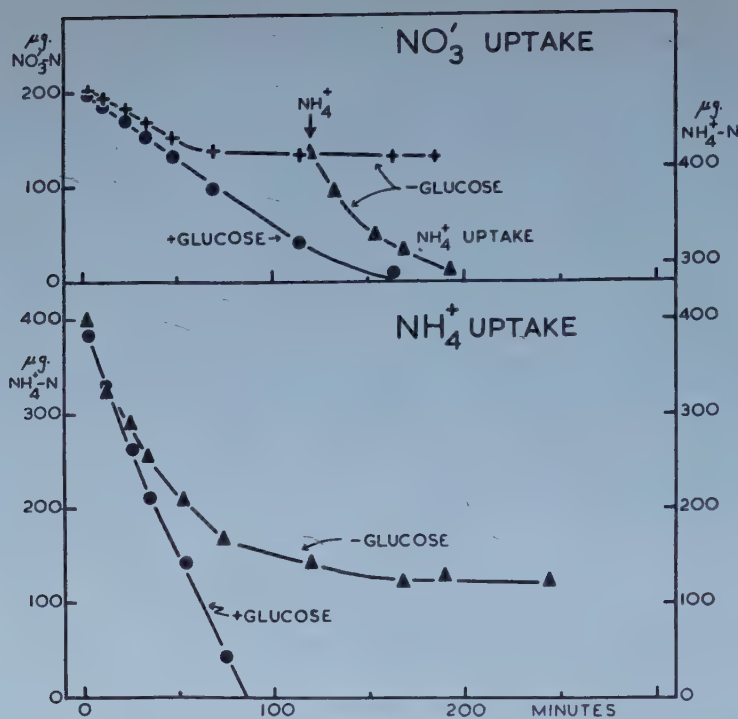
Figure 1. *Nitrate-N and ammonium-N assimilation by nitrogen-starved Chlorella with and without added glucose.* When the cells to which nitrate was added had ceased to assimilate nitrate, ammonium-N was added to them and its assimilation followed. The results are given as μg. N per 2.0 ml. of original suspension (2.5 ml. after additions); this contained 15.2 mg. dry wt. cells. Initial nitrogen concentration — 12 μ-atoms ammonium-N or 6 μ-atoms nitrate-N per ml. 0.04 *M* Glucose. Initial pH 6.19; 0.067 *M* phosphate.

ammonium-N was added to nitrogen-starved cells but showed little change when nitrate was added. When ammonium-N was added to the cells which had finished nitrate assimilation the soluble carbohydrate immediately dropped to the level of the ammonium cells.

This experiment, then, also leads to the conclusion that the cells are still nitrogen-starved when nitrate assimilation ceases since they still have the capacity of rapid ammonium-N assimilation. The problem is why these cells should cease to assimilate nitrate-N when they are still nitrogen-starved and still contain available carbohydrate whereas the addition of glucose allows nitrate assimilation to continue until all the nitrate has been assimilated.
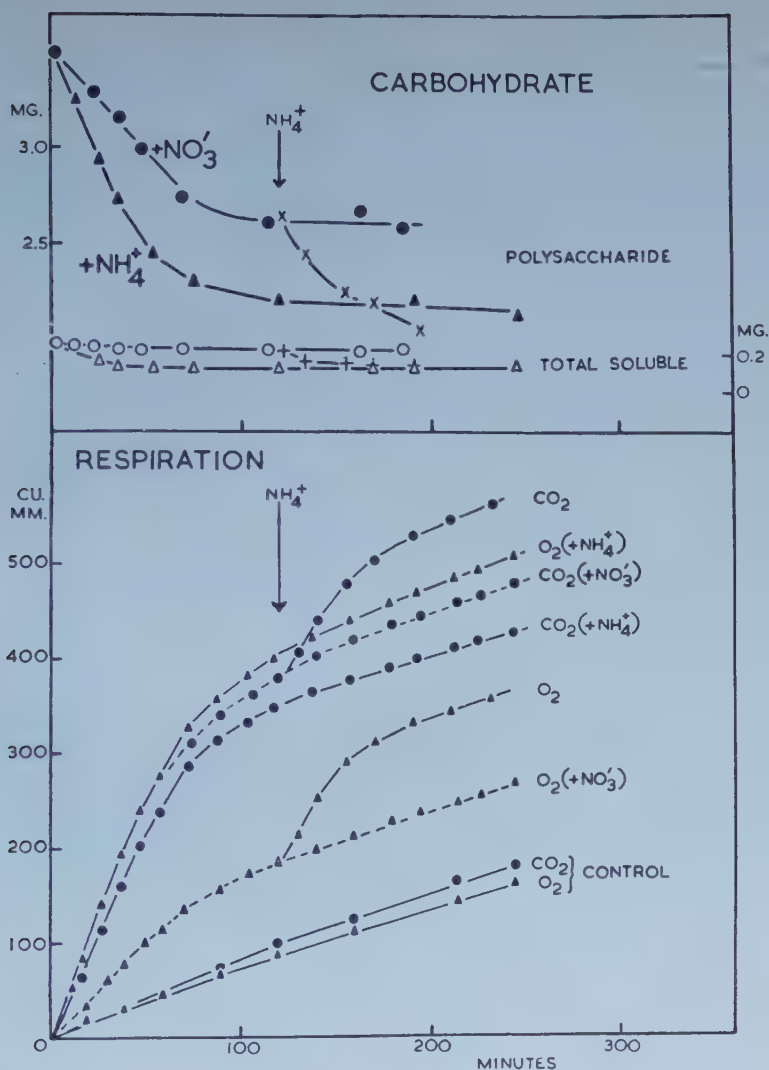
Figure 2. *Changes in gas exchange and intracellular carbohydrate following the addition of ammonium-N or nitrate-N to nitrogen-starved Chlorella.* When the cells to which nitrate was added had ceased to assimilate nitrate, ammonium-N was added to them. The results are given as μg. or μl. per 2.0 ml. of original suspension (2.5 ml. after additions); this contained 15.2 mg. dry wt. cells. For further details see legend to Figure 1. No glucose was added.

Possible explanations of the cessation of nitrate assimilation.

a) A change of the composition of the suspending medium — e.g. a drift of pH.

This possibility was eliminated by transferring cells which had ceased to assimilate nitrate to fresh suspending medium: they were still unable to assimilate nitrate but assimilated ammonium-N rapidly.

b) Trace element effects.

Although the cells were grown and starved of nitrogen in a medium containing trace elements (Fe, Cu, Mn, Mo, Zn), these elements were not added to the medium in which the cells were suspended for the experiments. The addition of these elements, however, either before nitrate was added or after nitrate assimilation had ceased had no effect on the nitrate assimilation.

c) Nature of intracellular polysaccharide.

It is possible that the intracellular polysaccharide consists of more than one fraction and that only part can be used for nitrate assimilation while all can be used for the assimilation of ammonium-N. Chromatograms of the hydrolysed polysaccharide show that this contains chiefly glucose and mannose. Glucose estimations, making use of the specificity of glucose oxidase, show that it is the glucose polysaccharide which is used up during nitrogen assimilation and this is so whether nitrate or ammonium-N is assimilated. (Figure 3). When nitrate is assimilated utilisation of the glucose polysaccharide stops before it is completely exhausted but it is metabolised again when ammonium-N is added. The residual component shows no change. This result is similar to that obtained by Yemm and Folkes (1954) with nitrogen-starved yeast which assimilated ammonium-N.

It is still possible that some glucose polymers can be used for ammonium-N assimilation but not for nitrate assimilation but this does not seem very likely.

d) Differences of oxidation-reduction potential between ammonium and nitrate cells.

Virtanen and v. Hausen (1949) observed that pea seedlings without cotyledons grew poorly in a nitrate medium unless a reducing substance such as glutathione or ascorbic acid was added. Similar seedlings grew well in an ammonium medium without any such addition. The explanation appears to be that the oxidation-reduction potential in the nitrate medium is too high for nitrate reduction by the seedlings. The potential is lowered by the addition of reducing agents which are presumably normally supplied from the cotyledons.

Figure 3. *Changes in glu-
cose polysaccharide fol-
lowing the addition of am-
monium-N and nitrate-N.
The results are given as
μg. per 2.0 ml. original
suspension. For further
details see legend to Fi-
gure 2.*
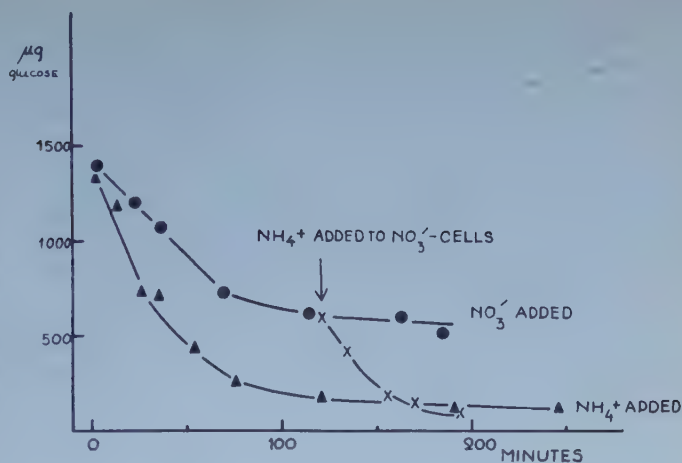
It seemed possible that a similar explanation applied to these experiments
with Chlorella. However, the addition of glutathione or ascorbic acid (40
μg./ml.) at pH 4.5 or 6.0 either before or after the cessation of nitrate assimi-
lation, was without effect. A difference of oxidation-reduction potential must
exist between the nitrate and ammonium cells and these experiments by
no means eliminate the possibility that this difference may be important in
determining the extent of nitrate assimilation.

As far as they have been tested, none of these possibilities explain the
cessation of nitrate assimilation satisfactorily. The data suggest that some
intermediate necessary for nitrate assimilation is supplied in sufficient quan-
tity from glucose during the whole of the assimilation period but from
endogenous polysaccharide for part of the period only. The nature of the
intermediate is unknown — perhaps it is the reduced form of a coenzyme.

Comparison of the rates of synthesis and respiration of nitrate and ammonium cells.

It was clear from previous experiments (Syrett, 1956) that it is chiefly
carbohydrate carbon which is converted to organic-N compounds when
nitrogen is assimilated by nitrogen-starved C. vulgaris. Since the amount of
carbohydrate which disappears is known, a balance sheet can be drawn up
showing how much intracellular carbohydrate has been converted to other
compounds and how much to carbon dioxide during the period of assimila-
tion. In Table 1 this has been done, for the experimental results of Fig. 2,
for the period 10—40 minutes after the addition of nitrate or ammonium-N
i.e. for the period of most rapid assimilation.

3

Table 1. *Changes during the period 10—40 minutes after the addition of ammonium-N or nitrate-N. No added glucose.* For experimental details see legend to Figure 2.

Data	Ammonium-N added	Nitrate-N added
Oxygen absorbed	158 µl.=7.05 µ-mol. O_2	61 µl.=2.72 µmol. O_2
Carbon dioxide produced	134 µl.=6.0 µ-atoms C	150 µl.=6.7 µ-atoms C
Carbohydrate loss (µg. equiv. glucose)	770 µg.=25.7 µ-atoms C	350 µg.=11.7 µ-atoms C
Ammonium-N assimilated	102 µg.	
Nitrate-N assimilated		36 µg.=2.57 µ-mol. NO_3'
µ-atoms H equivalent to O_2 (4H≡ 1 O_2)	28.2	10.9
µ-atoms H equivalent to NO_3' (8 H≡1 NO_3')	—	20.6
Total H equivalent	**28.2 µ-atoms**	**31.5 µ-atoms**
(1) Carbohydrate carbon loss	25.7 µ-atoms C	11.7 µ-atoms C
(2) Carbon lost as CO_2	6.0 µ-atoms C	6.7 µ-atoms C
(3) Carbohydrate carbon used in synthesis	19.7 µ-atoms C	5.0 µ-atoms C
Ratio $\frac{(3)}{(2)}$	$3.3 \frac{(19.7)}{(6.0)}$	$0.75 \frac{(5.0)}{(6.7)}$

It can be seen from this table that the nitrate cells produce rather more carbon dioxide but take up considerably less oxygen than the ammonium cells during this period. If allowance is made for the metabolic hydrogen ncessary to reduce nitrate to the reduction level of ammonia (8 atoms per nitrate ion) the amount of hydrogen which must be oxidized by oxygen in the ammonium cells is about the same as that oxidized in the nitrate cells by nitrate and oxygen together. In the nitrate cells there must be a competition between nitrate and oxygen for the hydrogen made available by the metabolism of carbohydrate. Since the rate of oxygen uptake hy the the nitrate cells is less than that of the ammonium cells it is probable that the concentration of reduced hydrogen acceptors such as $DPNH^+$ or $TPNH^+$ is maintained at a lower level in the nitrate cells. A similar inter-relationship between nitrate reduction and respiration has been described by Willis and Yemm (1955).

Table 1 also shows that whereas the nitrate cells produce rather more carbon dioxide, the ammonium cells convert considerably more carbohydrate to other compounds. In the ammonium cells 3.3 carbohydrate carbon atoms are converted to other compounds for every one which is lost as carbon dioxide. In the nitrate cells the ratio is only 0.75.

The greater rate of synthesis of nitrogenous compounds in the ammonium cells is reflected by changes in the concentration of soluble non-nitrogenous compounds. The soluble reducing substances rapidly decrease when ammo-

POLYSACCHARIDE $\xrightarrow{\quad B \quad}$ SOLUBLE ORGANIC -N $\xrightarrow{\quad C \quad}$ INSOLUBLE ORGANIC-N

$CO_2 \leftarrow$ | A

$O_2 \leftarrow$ [H]

NH_4^+

NO_3'
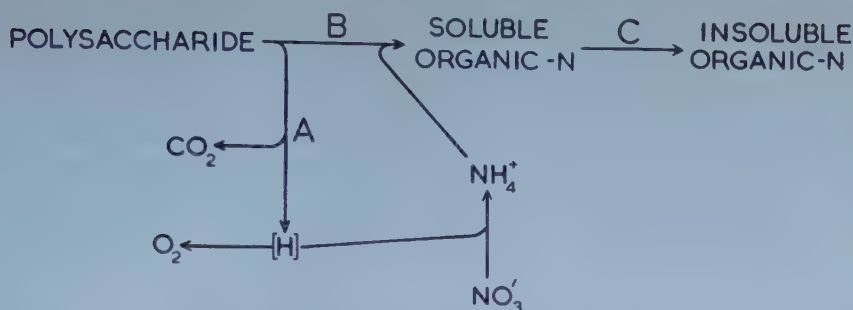
Figure 4. *Diagram of overall metabolism during nitrogen assimilation by nitrogen-starved cells of Chlorella.* See text for discussion.

nium-N is added but not when nitrate-N is added (Syrett, 1956). The concentration of α-ketoglutararte and pyruvate is lower in ammonium cells than in nitrate ones (Millbank, 1954). Evidently rapid synthesis in the ammonium cells removes non-nitrogenous substances from the metabolic pool more quickly than they are replaced from insoluble polysaccharide. In the nitrate cells, with less rapid synthesis, this does not happen.

Figure 4 depicts a very simplified scheme which attempts to link ammonium-N and nitrate-N assimilation by nitrogen-starved Chlorella cells. The reactions A, connected with respiration, appear to proceed at about the same rate in both nitrate and ammonium cells, but the synthetic reactions B proceed at quite different rates which are partly determined by the availability of reduced inorganic-N. This, in the nitrate cells, is determined to some extent, by the rate of reactions A. In the ammonium cells reactions B proceed much faster than C and soluble organic-N accumulates (Syrett, 1956); in the nitrate cells reactions B are slower and more nearly equal to C.

There is reason for believing that the rate of synthesis, B, partly controls the rate of respiration, A. (Syrett, 1953 b; Yemm and Folkes, 1954.) If this is so, the rate of supply of reducing hydrogen [H] from A will depend, to some extent, on the rate of nitrogen assimilation, and, in the nitrate cells, this will depend on the rate of nitrate reduction. But, since the rate of nitrate reduction is itself dependent on the rate of supply of [H], one can think of a metabolic cycle in the nitrate cells. Should the rate of reaction A decrease, possibly because of a drop in the concentration of polysaccharide, less [H] will be available, less nitrate will be reduced, less nitrogen will be assimilated and the respiration rate will drop still further, resulting in still less available [H]. In this way, a factor which caused only a slight slowing down of reaction A might very quickly stop nitrate assimilation completely. This may be the explanation of the cessation of nitrate assimilation by

nitrogen-starved cells before the polysaccharide is exhausted and while the cultures still contain nitrate. The addition of glucose, by supplying more [H] may allow assimilation to continue until all the nitrate has disappeared.

Such a cyclic scheme, with the rate of nitrate reduction and assimilation dependent on the supply of [H] which is, in turn, dependent on the rate of nitrate assimilation may account for the higher carbohydrate content of plants grown on nitrate in contrast to those grown with ammonium-N. (Street, 1949.)

However such a picture is clearly oversimplified and, while possibly depicting the overall metabolism, is not easy to interpret in terms of more detailed and quantitative biochemistry.

Summary

Nitrogen-starved cells of C. vulgaris cease to assimilate nitrate-N while they still contain available carbohydrate and the cultures still contain nitrate. When assimilation ceases the cells have apparently not recovered from nitrogen starvation since they will assimilate ammonium-N rapidly, and will continue to assimilate nitrate rapidly, if glucose is added.

Possible explanations of the cessation of nitrate assimilation are discussed and it is suggested that it may be connected with the interdependence of respiration, nitrate reduction and nitrogen assimilation.

When ammonium-N is assimilated 3.3 carbohydrate carbon atoms are converted to organic nitrogen compounds for every one which is lost as carbon dioxide. When nitrate is assimilated the ratio is 0.75. The data suggest competition between nitrate and oxygen for the hydrogen made available by carbohydrate breakdown.

References

Jermyn, M. A. & Isherwood, F. A.: Improved separation of sugars on the paper partition chromatogram. — Biochem. J. 44: 402. 1949.

Keilin, D. & Hartree, E. F.: The use of glucose-oxidase (Notatin) for the determination of glucose in biological material and for the study of glucose producing systems by manometric methods. — Biochem. J. 42: 230. 1948.

Millbank, J. W.: A study of intermediary metabolism in two micro-organisms with particular reference to keto-acids. — Ph. D. thesis, University of London. 1954.

Partridge, S. M.: Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. — Nature, Lond. 164: 443. 1949.

Street, H. E.: Nitrogen metabolism of higher plants. — Advanc. Enzymol. 9: 391. 1949.

Syrett, P. J.: The assimilation of ammonia by nitrogen-starved cells of Chlorelia vulgaris. Part I. The correlation of assimilation with respiration. — Ann. Bot. Lond. N.S. 17: 1. 1953 a.

— Part II. The assimilation of ammonia to other compounds. — Ann. Bot. Lond. N.S. 17: 21. 1953 b.

— The assimilation of ammonia and nitrate by nitrogen-starved cells of Chlorella vulgaris. II. The assimilation of large quantities of nitrogen. Physiol. Plant. 9: 19. 1956.

Virtanen, A. I. & v. Hausen, S.: Rôle of substances formed during germination in the growth of plants. Nature, Lond. 163: 482. 1949.

Willis, A. J. & Yemm, E. W.: The respiration of barley plants. VIII. Nitrogen assimilation and the respiration of the root system. New Phytol. 54: 163. 1955.

Yemm, E. W. & Folkes, B. F.: The regulation of respiration during the assimilation of nitrogen in Torulopsis utilis. Biochem. J. 57: 495. 1954.

Studies on the Connexion between Sucrose Formation and Respiration in Germinating Bean Cotyledons

By

YUKITO OOTA, RYOHEI FUJII and YOSHIO SUNOBE [1]

(Biological Institute, Nagoya University, Chikusa-ku, Nagoya, Japan)
(Received August 23, 1955)

Starch degradation in the germinating bean cotyledons is accompanied by simultaneous production of sucrose. In fact, the reserve form of carbohydrates of the cotyledons, i.e. starch, must be converted to the transport form, i.e. sucrose, before it is transferred to the growing parts of the seed embryos (Wanner, 1950 and 1952). This sugar production is likely closely dependent upon the presence of oxygen, implying an intimate relationship with oxygen respiration (Fujii, 1953; see also Porter, 1953 and 1955).

Recent studies in our laboratory have shown that in the early period of the germination stage [2] the cotyledon tissues of *Vigna sesquipedalis* have redox systems much or less specific to the facultative anaerobes, while those characteristic of aerobic organisms appear to be rather recessive. Thus in the bean cotyledons, cytochromes a and b, but not c, are present together with nitrate reductase, which probably functions as a terminal oxidase utilizing nitrate as the terminal hydrogen acceptor (Kumada, 1953; Yamamoto, 1954), whereas the TCA-cycle system is unlikely operative (Oota, Yamamoto and Fujii, 1953).

---

[1] Present address: Botanical Institute, Tokyo University, Hongo, Tokyo.

[2] Under our routine cultivation conditions, i.e. sand-culture at 30° C. in the dark, with no addition of nutrient except sufficient water, the growth (estimated as dry weight increase) of our materials. plantlets of Vigna sesquipedalis, ceases to proceed on the 7th day after the sowing due to the exhaustion of the cotyledon reserves; in the series of our studies on the growth of the bean seedlings it will thus be convenient to denote this 6-day period of heterotrophic growth of the seedlings by the term 'germination stage' (Oota, Fujii and Osawa, 1953).

It seems then worth studying as to how respiratory activity does participate, if it really does, in such an anabolic process as sugar formation accomplished in the germinating bean cotyledons whose metabolic function leans substantially, as has been repeatedly stressed, towards catabolism (cf. Oota, 1955).

In the present study, the effect of temperature on the changes in content of several carbon compounds including sucrose and, at the same time, on the gaseous exchange of the cotyledons excised from 1-day-old bean seedlings was investigated. Varying temperature had been expected to cause not only quantitative but also qualitative changes in the respiratory as well as the carbohydrate metabolism, and thus enable us to have a clue to the connexion between the sucrose formation and the respiration. The results obtained indicate that the cotyledon respiration may essentially be composed of two parts: carbohydrate and fat respirations, and that it would be the latter which may be associated with the sucrose formation. A small sucrose production was found to survive the exclusion of oxygen; no particular indication was given concerning the role played in the sugar formation mechanism by the above-mentioned respiratory systems of facultative anaerobe type.

Materials and Methods

Bean seeds. — Seeds of *Vigna sesquipedalis* stored in the dark for 3—6 months after harvest were used. They were soaked in water for 5 hrs. at 30° C. and grown for 24 hrs. in sand culture at 30° C. in the dark. The cotyledons were then detached from the embryos: they were selected for uniformity, washed repeatedly, and well blotted with filter paper. After being weighed they were placed in 50 ml. Erlenmeyer flasks. Each flask was equipped with a taper joint stopper bearing a stopcock and an inner cup; the inner cup contained a small amount of water to keep the gas phase moist. Although no particular asepsis was devised, the materials were kept practically free from bacterial contamination during the incubation period.

Gaseous exchange. — Conventional Warburg's respirometers were used for the estimation of oxygen absorption and carbon dioxide emission. Six well-blotted cotyledons (0.55 gm. in fresh weight) were placed in the main room of each conical vessel (ca. 27 ml. in volume). Mercury was employed as manometric fluid to cover marked pressure changes as experienced in the present study. The direct method was applied for carbon dioxide estimation (Umbreit, Burris and Stauffer, 1950).

In the anaerobic experiments, air was replaced with nitrogen by the procedure described by Umbreit et al. (1950). Cylinder nitrogen was washed before use by passing over heated copper and through a pyrogallol solution. The evacuation and nitrogen flushing procedure was repeated four times in 30 min. Before and after the incubation of 17-hr.-duration at designated temperatures the samples were withdrawn and analysed for the contents of following constituents.

Starch. — The method of Pucher et al. (1948) was applied. Starch was isolated

from 40 cotyledons (3.7 gm. f.w.) and an aliquot equivalent to 0.02 cotyledons was assayed.

Reducing and non-reducing sugars. — Alcohol extracts of the tissue homogenates made from 60 cotyledons (5.5 gm. f.w.) were cleared with lead acetate and an Amberlite IR-120 column, and an aliquot equivalent to 1.25 cotyledons was assayed for reducing sugars by the method of Somogyi (1952). Non-reducing sugars were estimated as the increase in reducing power by acid hydrolysis of an another aliquot of the alcohol extracts (equivalent to 0.125 cotyledons). By means of paper-chromatography it had been ascertained by Fujii (1956) that sugars contained in the alcohol extracts of Vigna sesquipedalis consist almost exclusively of sucrose together with a little glucose. Hence, the values of reducing and non-reducing sugars obtained were considered the indices of the contents of glucose and sucrose, respectively.

Ethyl alcohol. — The tissue homogenates made from 12 (1.1 gm. f.w.) or 24 (2.2 gm. f.w.) cotyledons were steam-distilled after being made alkaline to phenol red. The first 40 ml. distillate were assayed for ethyl alcohol by the method of Williams and Reese (1950) with slight modifications to fit to the large volume of the sample used. A known amount of alcohol was added as the carrier for distillation when needed. A possibility of contamination with aldehyde, if any, was not excluded in these estimations.

Lactic acid. — The tissue homogenates made from 12 cotyledons (1.1 gm. f.w.) were treated repeatedly with 10 per cent trichloroacetic acid, and an aliquot of the combined extracts (equivalent to from 0.24 to 0.96 cotyledons) was assayed for lactic acid by the Barker and Summerson's method as modified by Umbreit et al. (1950).

Lipid. — Sixty pieces of cotyledons (5.5 gm. f.w.) were ground with anhydrous sodium sulphate and the mixture was extracted with petroleum ether under a reflux for 8 hrs.; thus obtained lipid fraction was weighed after being dried at 95° C. to constant weight.

Results

Air experiments. — The effect of temperature ranging from 10 to 35° C. with a 5° C. interval on the changes in content of several carbon constituents and on the gaseous exchange of the cotyledons held in air was investigated. The results obtained are described in Table 1.

The contents of minor constituents, i.e. alcohol, lactic acid, and glucose, were little changed in the incubation period at every temperature examined, except that alcohol and lactate tended to increase with the rise in temperature over 30° C., and that the change in glucose passed an undefinite peak value at 20—25° C. The gaseous exchange and the starch loss showed marked growth with temperature. As to the change in sucrose two phases were clearly distinguished; thus the sugar content rose with temperature up to 25° C. and thereafter began to decline abruptly. Noteworthy was that the difference in quantity between the carbon in starch disappeared and that in sucrose formed dwindled with the falling temperature until it became nearly zero at 10° C. Thus at 10° C. the whole starch loss was apparently recovered as

Table 1. *The effect of temperature on the changes in content of various carbon compounds and on the gaseous exchange in the germinating bean cotyledons held in air in the dark. Carbohydrates are expressed as hexose equivalent. Each value represents at least 3 replicates; 0.95 confidence interval shown. Figures in parentheses indicate μmol. (mg. for lipid) increase (+) or decrease (−) in content per 17 hr. incubation.*

Substance determined	Initial content per cotyledon	Final content per cotyledon after 17 hr. incubation at:					
		10° C.	15° C.	20° C.	25° C.	30° C.	35° C.
Starch (mg. hexose)	19.9 ± 0.29	19.6 ± 0.50 (−1.67)	19.2 ± 0.24 (−3.88)	18.8 ± 0.22 (−6.11)	18.0 ± 0.28 (−10.0)	17.3 ± 0.35 (−14.4)	16.8 ± 1.15 (−17.2)
Sucrose (mg. hexose)	1.023 ± 0.051	1.417 ± 0.286 (+2.19)	1.604 ± 0.126 (+3.23)	1.848 ± 0.331 (+4.58)	2.353 ± 0.041 (+7.39)	2.086 ± 0.204 (+5.90)	1.764 ± 0.141 (+4.12)
Glucose (mg.)	0.086 ± 0.001	0.081 ± 0.013 (−0.03)	0.101 ± 0.064 (+0.08)	0.148 ± 0.018 (+0.34)	0.153 ± 0.191 (+0.37)	0.133 ± 0.000 (+0.26)	0.098 ± 0.013 (+0.07)
Ethyl alcohol (μg.)	22.9 ± 1.81	21.0 ± 12.7 (−0.04)	23.0 ± 8.96 (+0.02)	25.2 ± 6.34 (+0.05)	27.8 ± 6.70 (+0.11)	36.7 ± 9.60 (+0.30)	57.0 ± 4.72 (+0.74)
Lactic acid (μg.)	27.8 ± 5.95		32.8 ±12.6 (+0.06)		25.5 ± 8.43 (−0.03)		51.3 ±18.4 (+0.26)
CO_2 output (μl.)		82 ± 5.03 (+3.65)	192 ±15.2 (+8.55)	360 ± 9.80 (+16.1)	489 ±31.5 (+21.8)	574 ±22.4 (+25.6)	643 ±16.0 (+28.7)
O_2 uptake (μl.)		116 ± 7.85 (−5.18)	231 ±14.4 (−10.3)	420 ±27.3 (−18.7)	562 ±30.5 (−25.0)	651 ±20.7 (−29.1)	707 ±19.4 (−31.5)
Lipid (mg.)	0.500 ± 0.029		0.420 ± 0.076 (−0.080 mg.)		0.314 ± 0.021 (−0.186 mg.)		0.380 ± 0.222 (−0.120 mg.)

Table 2. *Separation of the cotyledon respiration into its two components: carbohydrate and fat respirations.*

Process	Respiratory O_2 uptake (μmol O_2/17 hrs./cotyledon) at:					
	10° C.	15° C.	20° C.	25° C.	30° C.	35° C.
Overall respiration .... (R.Q. =)	5.18 (0.706)	10.3 (0.830)	18.7 (0.856)	25.0 (0.870)	29.1 (0.882)	31.5 (0.910)
Carbohydrate respiration... (R.Q. = 1.00)	0.08	4.60	10.0	14.3	17.4	22.2
Fat respiration .... (R.Q. = 0.70)	5.10	5.70	8.66	10.7	11.7	9.30

sucrose produced. It should also be noticed that the respiratory quotient (R.Q.) was depressed with the drop in temperature: from 0.910 at 35° C. to 0.706 at 10° C. (Table 2). The R.Q. obtained at 10° C., 0.706, implies the sole occurrence of the fat respiration at that temperature. From this a close connexion between the sucrose formation and the fat respiration may be inferred to exist. Assuming that under the present conditions the bean cotyledons respire exclusively two substrates, i.e. carbohydrate (R.Q. = 1.00) and fat (R.Q. = 0.70), the share taken by each component in the respiratory gas exchange at respective temperatures was computed (Table 2). Interesting to say, at the temperature range, 10—25° C., the theoretical amounts of respired carbohydrate (μmol $O_2 \cdot \frac{1}{6}$ consumed by the carbohydrate respiration as shown in Table 2) were found to agree very well with the differences between the starch loss and the sugar gain, i.e. in Table 3 both items being compared on a hexose basis, no statistically significant discrepancy was detected at least in the above-mentioned temperature range. This seems to justify the above postulation of the dual composition of the cotyledon respiration.

An anomaly, however, was found at 30 and 35° C., where as shown above an evident decay in the net synthesis of sucrose took place without any concurrent depression in the starch loss. Thus at these temperatures the difference between the starch loss and the sugar gain was too large to be

Table 3. *Comparison of the carbohydrate respired with the difference between the starch loss and the sucrose formed.* Carbohydrate respired = carbohydrate respiration (μM O_2 from table 2) $\times \frac{1}{6}$.

Carbohydrate tranformation	Changes in carbohydrates (μmol hexose/17 hrs./cotyledon) at:					
	10° C.	15° C.	20° C.	25° C.	30° C.	35° C.
Carbohydrate respired	0.01	0.77	1.68	2.38	2.90	3.70
Starch loss minus sucrose formed	0(?)	0.66	1.53	2.60	8.50	13.1

Table 4. *The effect of temperature on the changes in content of various carbon compounds and on the gaseous exchange in the germinating bean cotyledons held in nitrogen in the dark.* Carbohydrates are expressed as hexose equivalent. Each value represents at least 3 replicates; 0.95 confidence interval shown. Figures in parentheses indicate μmol. (mg. for lipid) increase (+) or decrease (—) in content per 17 hr. incubation.

Substance determined	Initial content per cotyledon	Final content per cotyledon after 17 hr. incubation at:		
		15° C.	25° C.	35° C.
Starch (mg. hexose) ...	20.8 ± 0.219	20.1 ± 0.622 (— 3.89)	19.0 ± 1.280 (— 10.0)	19.0 ± 0.582 (— 10.0)
Sucrose (mg. hexose)...	1.072 ± 0.048	1.178 ± 0.201 (+ 0.59)	1.368 ± 0.075 (+ 1.65)	0.852 ± 0.191 (— 1.22)
Glucose (mg.)	0.084 ± 0.029	0.080 ± 0.013 (— 0.02)	0.079 ± 0.008 (— 0.03)	0.075 ± 0.064 (— 0.05)
Ethyl alcohol (μg.)	21.0 ± 2.95	105 ± 8.80 (+ 1.83)	230 ± 13.2 (+ 4.54)	267 ± 29.3 (+ 5.33)
Lactic acid (μg.).........	26.9 ± 3.48	131 ± 12.5 (+ 1.15)	241 ± 23.6 (+ 2.37)	325 ± 27.4 (+ 3.31)
CO_2 output (μl.)		98 ± 7.12 (+ 4.37)	332 ± 10.1 (+ 14.8)	619 ± 9.22 (+ 27.6)
Lipid (mg.)	0.520 ± 0.013	0.480 ± 0.010 (— 0.040 mg.)	0.444 ± 0.010 (— 0.076 mg.)	0.447 ± 0.044 (— 0.073 mg.)

ascribed to the calculated amount of carbohydrate respired (Table 3). The production of alcohol and lactic acid, if both were derived from starch, was so small that it can not be responsible for the unidentified proportion of the starch loss. These features of metabolic activities shown at 30—35° C., i.e. depressed sucrose formation, enhanced alcohol and lactate production, appeared to imply the occurrence of a partial anaerobiosis in the tissues in question. This notion is to be verified by the nitrogen experiments which will be described in next sub-section.

On the other hand, the evaluated share of the fat respiration, having a peak value at 25° C., was found just to keep pace with the sucrose formation (Tables 1 and 2). The carbohydrate respiration was accelerated rather simply with the rise of temperature (Tables 1 and 2), and no correlation was seen with the sucrose formation. Table 1 contains another evidence favourable to the interrelation between carbohydrate and fat metabolism: the tissues lose their lipid much or less rapidly, the extent of the lipid disappearance being nearly parallel with that of the sucrose formation.

Nitrogen experiments. — Secondly, the effect of temperature, 15, 25 and 35° C., on the carbon metabolism of the cotyledons placed under nitrogen was investigated. The results obtained are shown in Table 4. Inspection of the table reveals the distinctive features of the anaerobic pattern of the

cotyledon metabolism, i.e. marked production of alcohol and lactate, dimi-
nution in sugar formation, emission of an excess of carbon dioxide and
surplus starch loss. It must be noticed that nearly identical situations, though
quantitatively somewhat modified by the superposition of the aerobic pattern,
were observable in the air experiments conducted at the temperature condi-
tions above $30°$ C. The higher the temperature rose, the stronger was the
alcohol and lactic acid production. The rate of decrease in starch content,
however, was enhanced with temperature only up to $25°$ C., and, thereafter,
remained unchanged.

By the removal of oxygen the net synthesis of sucrose at 15 and $25°$ C.
was decreased by about 80 per cent, thus one fifth of the aerobic sucrose
production survived the oxygen elimination. Turner (1954) has demonstrated
with his partially purified pea enzyme system that sucrose synthesis is
reduced by just the same extent, i.e. 80 per cent, when oxygen is replaced
by nitrogen. Hence the remaining activity of synthesis detected in the bean
cotyledons held in nitrogen may not be due to an inadequate elimination of
oxygen in the tissues in question. At $35°$ C. the initial level of the sucrose
content was decreased markedly in the incubation period. This would suggest
preferential utilization of sugar at higher temperatures probably without
accompanying *de novo* synthesis of sucrose.

At temperatures below $25°$ C., the starch diminution apparently indepen-
dent of the gas conditions was neteworthy. Thus either in nitrogen or in
air the starch degradation at 15 and $25°$ C. amounted to 3.9 and 10 µmol
per cotyledon per 17 hrs., respectively. Just as in the case of starch disappear-
ance, the rate of lipid disappearance increased with the temperature up
to $25°$ C. to attain a constant value. No change in lipid, alcohol, or lactic
acid was thus detected which could clearly be correlated with that in
sucrose.

At any temperature examined the carbon contained in starch degraded
exceeded appreciably the combined quantity of the carbon in sucrose, alco-
hol, lactic acid, and carbon dioxide formed. As to the carbon dioxide produc-
tion it was far beyond the requirements of the equation of alcoholic fermen-
tation, i.e. the molecular ration: alcohol/carbon dioxide « 1.

Observed changes in the glucose content represented only unsignificant
proportion of the anaerobic carbon traffic.

Discussion

In germinating seeds three major categories of reserve nutrients, i.e.
starch, fats and proteins, are known to be utilized as respiratory substrates

not always concurrently, but usually in turn in an order likely specific of a given plant (cf. Stiles and Leach, 1952; James, 1953). The following evidences indicate preferential utilization of both fats and starch in the normal respiration process of the present materials. Noda (1955) in our laboratory has recently shown that the level of soluble amino-nitrogen (113 µg. N/cotyledon) in the cotyledons which were excised from the axial tissues at the onset of germination and incubated in a dark, moist chamber at 30° C. is elevated in a day to a new level (150 µg. N) which is maintained unchanged for subsequent days, whereas in the cotyledons of intact seeds the level is depressed rapidly after a similar temporary rise up to a little higher value (175 µg. N) (see also Oota, Fujii and Osawa, 1953). With a separate experiment we have found that the same materials as used in the present study can be stored overnight at 25° C. in the dark without any appreciable loss in the protein content, whereas almost linear decrease starts from the very beginning of germination in the cotyledons attached to the intact seedlings (see also Oota, Fujii and Osawa, 1953). As to starch and fat disappearance in the cotyledons, it was shown separately that the processes could go on vigorously without the growing tissues attached, not to speak of the disappearance in the presence of the growing tissues attached. Thus in the bean cotyledons starch and fats are considered to be degraded to smaller molecules to be partly transported to the growing parts of the embryos, and partly consumed in the respiratory process of the cotyledons. In contrast to this, the protein degradation will already be stopped at the level of amino acids and amides which are solely transferred to the axial tissues (Yamamoto, 1955), but not respired in the cotyledons. Thus in the cotyledons isolated from the growing tissues the breakdown of proteins will be blocked before long by the accumulation of the hydrolysis products, while the starch and lipid degradation will proceed without ceasing as long as the products are removed from the site of reaction by there operating respiratory process.

Concerning the metabolic connexion between fats and carbohydrates, the possibility of fat-sugar interconversion must first be examined. As is known for oily seeds, sucrose can be produced from fats via hexoses according to the following equilibria (cf. James, 1953):

$$\text{I} \qquad \text{II}$$
$$\text{fat} \rightleftarrows \text{hexose} \rightleftarrows \text{sucrose}$$

With the rise in temperature, the equilibrium I that is exothermic may shift to left, while the endothermic process of the hexose-sucrose conversion (II) may be hastened. The over-all fat-sucrose process may thus be limited by the equilibrium II at lower temperatures and by I at higher temperatures. Accordingly, if this fat-sucrose conversion is the sole process of sucrose formation operating in the cotyledons, the sucrose-temperature curve can have a peak value at a certain temperature, as is actually the case. This hypothesis, though plausible, rejects further examina-

tions of its validity owing to the lack in detailed informations on the equilibria I and II, and, moreover. provides no explanation for the drift of R.Q. disclosed with temperature.

The analysis of the R.Q. drift favors strongly the alternative hypothesis, which assumes that sucrose will be derived from starch as anticipated at the opening of this paper, and the process would be associated, may be calorifically, with the respiratory oxidation of fats. In particular, the carbon balance sheet compiled at 10° C., where the R.Q. is 0.706, suggests that at this temperature starch may be utilized exclusively for the production of sucrose, being backed up by the fat respiration. On the other hand, it has been pointed out in the preceding section that the symptoms of the cotyledon metabolism in hot air is comparable with that observed under anaerobic conditions. Thus the rate of sucrose synthesis in the cotyledons which increases with temperature up to 25° C., may decrease thereafter as the rising temperature induces increasing oxygen shortage in the tissues. As described by Burton (1950) concerning the effect of storage temperature upon the oxygen content of the potato tuber, this partial anaerobiosis induced by higher temperatures may also readily be explained in terms of an unbalance between consumption and supply of oxygen due to the high temperature coefficient of respiration and the reduced solubility of oxygen in the cell sap.

Correlation coefficients were determined between the sucrose production and the fat respiration and the lipid loss. The sucrose production was thus confirmed to be well correlated to the fat respiration ($r = 0.887$, d.f. $= 4$; $P < 0.05$), but not to the total lipid loss ($r = 0.997$, d.f. $= 1$; $P > 0.05$). The lipid, being separated as the petroleum ether soluble fraction, may be of highly complex composition, including phospholipids (Sunobe, 1954) etc. besides fats, and as shown below even the glycerol moiety of fat may probably be not involved in the sucrose synthesis. This would be the reason why a comparatively lower value was obtained between the sugar synthesis and the lipid loss.

Recently, Stumpf and his collaborators have succeeded in providing the first satisfactory explanation as to the mechanism of respiratory oxidation of higher fatty acids such as palmitic acid (Newcomb and Stumpf, 1952: Humphreys, Newcomb, Bokman and Stumpf, 1954). According to these authors, the oxidation of these fatty acids to carbon dioxide in germinating peanut cotyledons may be catalysed by two systems localized in the microsomal particles and the soluble cytoplasmic proteins, and, in striking contrast to the common view of animal and microbe biochemists, no participation of the mitochondrial particles. It appears not improbable that these nonmitochondrial fractions can perform almost complete, if not complete, oxidation of the acids in the presence of required cofactor(s). In this connexion, it should be mentioned that the combustion quotient (C.Q.), i.e. the ratio

CO_2/O_2 for the complete oxidation, of higher fatty acids common to legume plants, e.g. linolenic, oleic and palmitic acids (cf. Bonner, 1950) always lie in the proximity of 0.70 in agreement with the R.Q. assumed for the fat respiration of the cotyledons. No data, however, are available at present as to whether these legume acids other than palmitic acid are also attacked by the non-mitochondrial fractions. The oxidation of the glycerol moiety is said to involve the mitochondrial enzyme including the complete TCA-cycle system as well as a non-particulate system of soluble proteins (Stumpf, 1955). The C.Q. of glycerol is 0.86.

On the other hand, concerning the subcellular locus of sucrose formation, Turner (1953) has demonstrated that the synthetic activity of pea is not sedimentable by centrifugation at 20,000 g for 30 min.; accordingly here also may be no participation of mitochondria. A probability must therefore be stressed that the aerobic sucrose synthesis in the cotyledon tissues would be carried out without the participation of mitochondrial activities.

As to the sucrose formation in anaerobiosis, nothing as yet can be said on its energetical relation. No evidence positively in support of Turner's postulation (Turner, 1954) of an energetical connexion between the glycolytic system and the sucrose formation has been obtained in the present study, nor any fact indicating the role played by the respiratory system of facultative anaerobe type.

In any case, the interrelationship deduced between the sucrose formation and the fat respiration is to be reestablished with the subcellular fractions excluding mitochondria, and the question must also be answered of whether the connexion is ubiquitous in plant tissues or only exceptional in storage tissues of catabolic nature.

The statement of Stumpf (1955) that the TCA-cycle may be operative in regard of the glycerol oxidation in the germinating peanut cotyledons sounds to be contradictory to our conviction on the absence of this metabolic dynamo of highest efficiency in the germinating bean cotyledons. It has been pointed out that the present materials can have no citric dehydrogenase activity by lack of triphosphopyridine nucleotide (TPN) (Oota, Yamamoto, and Fujii, 1953). Marrè and Servettaz (1954) must here be quoted. Studying the activity-ratio between TPN-linked enzymes (glucose-6-phosphate and citric) and diphosphopyridine nucleotide (DPN)-linked ones (malic and pyruvic) in pea seedlings, they have come to a conclusion that the dominancy of the former systems is regarded as characteristic of tissues endowed with the ability of protoplasmic growth; 'static' tissues including cotyledons are deficient in the TPN-linked enzymes. It appears to be a problem of special interest whether the TCA-cycle system is really operative or not in the germinating seed cotyledons.

The experiments in nitrogen have been performed so as not to provide a precise picture of the anaerobic pattern of the cotyldon metabolism, but only to provide clue to the anomaly appeared in the aerobic pattern when

the temperature was elevated above 30° C., and the cotyledon tissues were thus found to behave very similarly in hot air and in nitrogen. Nevertheless, some aspects of the results obtained in the anaerobic experiments merit further discussion.

As to the alcohol production in plant tissues, the fermentation system of yeast type is thought to be of wide distribution (cf. Stumpf, 1952; Stiles and Leach, 1952; James, 1953). We have, however, at present no particular evidence for the presence of the zymase system in the bean cotyledons excepting that highly active alcohol dehydrogenase has been detected in the tissues in question (Oota, Yamamoto and Fujii, 1953).

The prevalent occurrence of lactic acid in higher plants, in particular, held in anaerobiosis, has been discussed by several authors (e.g. Barker and Saifi, 1952 a and b). And the reaction sequence analogous to the muscle glycolysis is thought to be responsible for the lactate production in plants. Oota, Yamamoto and Fujii (1953), however, were unable to find any indication of the presence of lactic dehydrogenase in the cotyledon extracts by means of Thunberg's technique. Hence, although a negative result proves little, it may still be premature to assume the existence of the glycolytic system in the present materials.

At least at moderate temperatures, the striking agreement was noticed between the starch loss in air and the one in nitrogen. In air, however, the net loss in carbohydrates was reduced by about 75 per cent of the total starch loss, due mainly to the vigorous sucrose formation. Thus in the present materials was evidently demonstrated 'the action of oxygen in diminishing carbohydrate destruction and in supressing or decreasing the accumulation of the products of anaerobic metabolism', that is to say, the operation of the Pasteur effect (Dixon, as cited by Turner, 1951). Although the precise mechanism of the sucrose synthesis in higher plants is yet to be determined, it would be reasonable to assume that in the cotyledons starch must first be decomposed to hexoses to be back-synthesized into sucrose. Hence a sort of oxidative back-synthesis, or oxidative anabolism in a broader sense than the original use of Blackman (1928), is thought to be involved in the aerobic conversion of starch to sucrose. Thus the Pasteur effect operating in the germinating bean cotyledons involves oxidative anabolism in its mechanism. It appears to be of special significance that here the Pasteur effect provides this catabolic organ with the device of transformation of carbohydrates from the reserve form to the transport one; needless to say, the significance of the Pasteur effect in normal, growing tissues should be sought in its contribution to the anabolic work of body construction.

The complexity of the anaerobic pattern, unidentified portion in the carbon traffic, will be dealt with in detail with future experiments.

Summary

The effect of temperature ranging from 10 to 35° C. on the changes in content of starch, sucrose, glucose, ethyl alcohol, lactic acid, and lipid, and also on the gaseous exchange of the cotyledons separated from germinating seeds of *Vigna sesquipedalis* was investigated.

Starch loss and respiration were enhanced exponentially with the rise in temperature, whereas as for sucrose formation a distinct peak value at 25° C. was observed. The R.Q. was depressed from 0.91 to 0.71 in correspondence with the drop in temperature from 35 to 10° C. At 10° C. the starch loss was apparently just recovered as the sucrose gain. Changes in the lipid content took place in an inverse proportion to that in the sucrose content.

These results being explained by assuming the dual composition of the cotyledon respiration, i.e. carbohydrate (R.Q. = 1.00) and fat (R.Q. = 0.70) respirations, it was deduced that in the storage tissues in question the oxidative back-synthesis of sucrose, as the major translocation form of carbohydrates, might occur in a close connexion with the fat respiration.

Under the temperature conditions above 30° C., a little formation of alcohol and lactate was detectable besides the depression in sucrose formation.

Experiments otherwise similar to the aerobic ones were performed under nitrogen, and the anaerobic pattern of the cotyledon metabolism was revealed to be characterized by the concurrent formation of ethyl alcohol and lactic acid accompanied by a slight sucrose production. Thus, anomalies observed in hot air above 30° C. were ascribed to a temperature induced partial suffocation of the tissues in question.

References

Barker, J. & el Saifi, A. F.: Studies in the respiratory and carbohydrate metabolism of plant tissues. I. Experimental studies of the formation of carbon dioxide, lactic acid and other products in potato tubers under anaerobic conditions. — Proc. Roy. Soc. B 140: 362. 1952 a.

— & —: Studies in the respiratory and carbohydrate metabolism of plant tissues. II. Interrelationship between the rates of production of carbon dioxide, of lactic acid and of alcohol in potato tubers under anaerobic conditions. — Proc. Roy. Soc. B 140: 385. 1952 b.

Blackman, F. F.: Analytic studies in plant respiration. — Cambridge Univ. Press, Cambridge, 1954.

Bonner, J.: Plant Biochemistry. — Academic Press, New York, 1950.

Burton, W. G.: Studies on the dormancy and sprouting of potatoes. I. The oxygen content of the potato tuber. — New Phytol. 49: 121. 1950.

Fujii, R.: 1953. (Unpublished.)

Fujii, R.: Paper-chromatographic survey of soluble sugars in germinating bean seed embryos. — Bot. Mag. (Japan) 1956. (In press.)

Humphreys, T. E., Newcomb, E. H., Bokman, A. H. & Stumpf, P. K.: Fat metabolism in higher plants. II. Oxidation of palmitate by a peanut particulate system. — J. Biol. Chem. 210: 941. 1954.

James, W. O.: Plant respiration. — Clarendon Press, Oxford, 1953.

Kumada, H.: The nitrate utilization in seed embryos of Vigna sesquipedalis. — J. Biochem. (Japan) 40: 439. 1953.

Marrè, E. & Servettaz, O.: Ricerche sulla fisionomia respiratoria caratteristica di tessuti in fase di attiva crescita protoplasmatica. Deidrogenasi a TPN e deidrogenasi a DPN. — Rend. Acc. Lincei 16: 521. 1954.

Newcomb, E. H. & Stumpf, P. K.: Fatty acid synthesis and oxidation in peanut cotyledons. — Phosphorus metabolism. Vol. 2., Johns Hopkins Press, Baltimore, 1952.

Noda, Y.: 1955. (Unpublished.)

Oota, Y., Fujii, R. & Osawa, S.: Changes in chemical constituents during the germination stage of a bean, Vigna sesquipedalis. — J. Biochem. (Japan) 40: 649. 1953.

Oota, Y., Yamamoto, Y. & Fujii, R.: Dehydrogenase pattern in bean seed embryo. — J. Biochem. (Japan) 40: 187. 1953.

Oota, Y.: Biochemistry of seed germination. — Biochemistry of life phenomena (In Japanese), Asakura Publish. Co., Tokyo, 1955.

Porter, H.: Starch synthesis and degradation in vivo. — Biochem. Soc. Symp. 11: 27. 1953.

— & May, L. H.: Metabolism of radioactive sugars by tobacco leaf disks. — J. Exptl. Bot. 6: 43. 1955.

Pucher, G. W., Leavenworth, C. S. & Vickery, H. B.: Determination of starch in plant tissues. — Anal. Chem. 20: 850. 1948.

Somogyi, M.: Notes on sugar determination. — J. Biol. Chem. 195: 19. 1952.

Stiles, W. & Leach, W.: Respiration in plants. — Methuen & Co., London, 1952.

Stumpf, P. K.: Glycolytic enzymes in higher plants. — Ann. Rev. Plant Physiol. 3: 17. 1952.

— Fat metabolism in higher plants. III. Enzymic oxidation of glycerol. — Plant physiol. 30: 55. 1955.

Sunobe, Y.: (Unpublished.)

Turner, J. F.: Synthesis of sucrose by a plant enzyme extract. — Nature 172: 1149. 1953.

— Synthesis of sucrose by a partially purified plant tissue extract. — Nature 174: 692. 1954.

Turner, J. S.: Respiration. The Pasteur effect in plants. — Ann. Rev. Plant Physiol. 2: 145, 1951.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: Manometric techniques and tissue metabolism. — Burgess Publish. Co., Minneapolis, 1950.

Wanner, H.: Über die Zuckerwanderung in Erbsenkeimlingen. — Ber. Sweiz. Bot. Ges. 60: 426. 1950.

— Natur und Verteilung der löslichen Kohlehydrate im Erbsenkeimling. 2. Qualitative und quantitative Bestimmung der einfachen Zucker durch Papierchromatographie. — Ber. Schweiz. Bot. Ges. 62: 205. 1952.

Williams, M. B. & Reese, H. D.: Colorimetric determination of ethyl alcohol. — Anal. Chem. 22: 1556. 1950.

Yamamoto, Y.: On formic dehydrogenase of bean seed embryo in the germination stage. — J. Biochem. (Japan) 41: 551. 1954.

Yamamoto, Y.: Asparagine metabolism in the germination stage of a bean, Vigna sesquipedalis. — J. Biochem. (Japan) 1955. (In press.)

Further Studies on the Effect of Various Cycles of Light and Darkness on the Growth of Tomato and Cocklebur Plants

By

Erik K. Bonde [1]

Division of Biology, California Institute of Technology
(Received October 21, 1955)

Introduction

In a previous paper (Bonde 1955) data were presented showing the effects of alternating periods of light and darkness of equal length on the growth of young tomato and cocklebur plants over a period of from 2 to 3 weeks. The present work is a continuation of the above study in that the effects of various other cycles, mostly shorter, were studied. In addition attempts were made to modify the growth patterns under the different cycles through the application of various substances to the leaves of the plants in the form of sprays. In this paper also, as an example, a cycle of 1 hour of light and 1 hour of darkness will be called a "1-hour cycle", the alternating light and dark periods in each treatment always being of the same length.

Materials and Methods

As described in detail in the previous paper, young seedlings of tomato and cocklebur were grown in vermiculite under controlled conditions for about 3 weeks before being selected into groups of 9 for testing with alternating periods of light and darkness in "light boxes". During the course of the

---

experiments reported in this paper the sliding covers of the light boxes were replaced by louvers which could also be automatically opened and closed to admit or exclude light from the bank of fluorescent tubes interspersed with incandescent bulbs above the row of boxes. In these experiments only a single intensity of 1000 foot-candles was used, since no great differences appeared in the growth patterns with different intensities in the earlier work.

Three types of sprays were employed in this study: 10 per cent solution of sucrose in water, 5×10^{-4} M solutions of indoleacetic acid (IAA), and 0.5 and 5.0 mg/l solutions of 2,3,5-triiodobenzoic acid (TIBA). A small amount of spreading agent (Tween 80) was added to each solution. Sucrose solution was applied daily to the leaves of the plants, the IAA every three days, and the TIBA once at the beginning of the experimental period. In each case the leaves were thoroughly wetted by means of a small hand sprayer. Young cocklebur and tomato plants were tested with the various sprays in combinations of 6-second, 1-minute, 5-minute, 30-minute, 1-hour, and 2-hour cycles.

Results

Effects of various cycles on increase in dry weight

The "Control" curve in Figure 1 shows the effects on cocklebur plants of short cycles (5-minute, 30-minute, 1-hour and 2-hour) during a 19-day test period on the increase in dry weight of the plant tops. Here the 1-hour cycle produced the best growth, with the 5-minute and 2-hour cycles being somewhat poorer and the 30-minute cycle very poor. The plants under the 1-hour cycles produced more than 3 times as much increase in dry weight as those under the 30-minute cycles. The effects of still shorter cycles (6-second, 1-minute, 5-minute, 30-minute, and 1-hour) on the cocklebur are shown by the "Control" curve of Figure 2. In this series the 1-hour cycles were not markedly superior to the 30-minute cycles in effect, but the growth under the 1-minute cycles was 3 times as great as that under the 5-minute cycles, and the growth under the 6-second cycles also was greater than under the 5-minute, 30-minute, and 1-hour cycles.

The "Control" curve in Figure 3 shows the effects on dry weight increase in a series of tomato plants grown for 17 days under 5-minute, 30-minute, 1-hour, and 2-hour cycles. In this particular series the differences in dry weight increase were not great. The increase was largest under the 5-minute cycles, smallest under the 30-minute cycles, and intermediate under the 1-hour and 2-hour cycles. These differences may be seen more clearly in the "Control" curve of Figure 4, which shows the results of a series consisting

Figure 1. *Average increase in dry weight in 19 days of tops of cocklebur plants grown at 1000 foot-candles under various cycles of light and darkness. Sucrose was applied daily as a 10 per cent spray; IAA was applied every 3 days as a 5×10^{-4} M spray.*
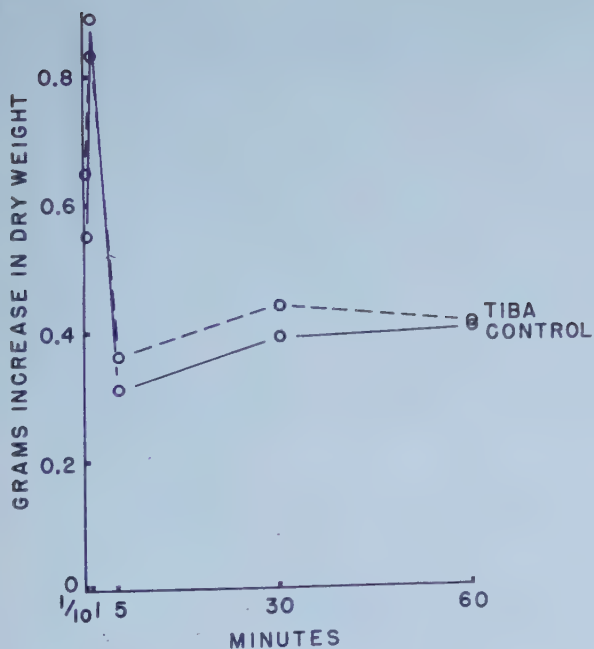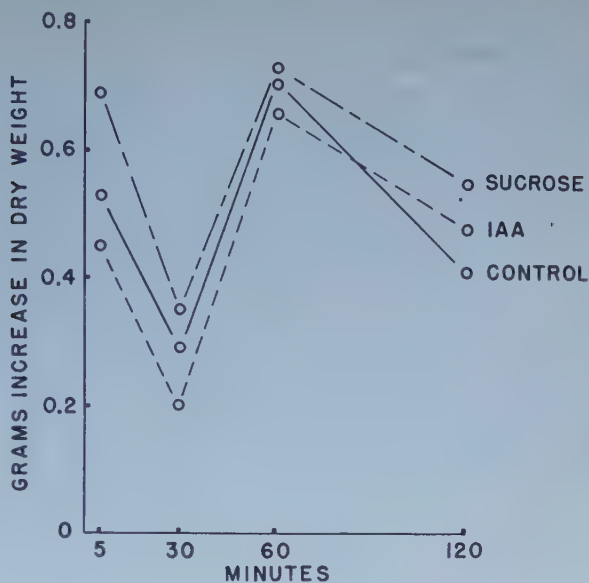
Figure 2. *Average increase in dry weight in 13 days of tops of cocklebur plants grown under various cycles of light and darkness. 5.0 mg/l of TIBA was applied as a spray at the beginning of the experimental period.*
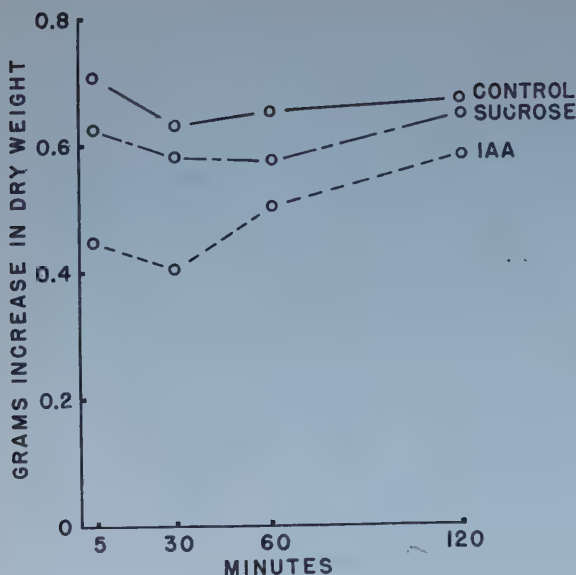
Figure 3. *Average increase in dry weight in 17 days of tops of tomato plants grown under various cycles of light and darkness. Sucrose was applied daily as a 10 per cent spray; IAA was applied every 3 days as a 5×10^{-4} M spray.*

of 6-second, 1-minute, 5-minute, 30-minute, and 1-hour cycles tested for 12 days. Here the greatest dry-weight increase was produced under the 6-second cycles, with less under the 1-minute, somewhat more again under the 5-minute, and considerably less under the 30-minute and 1-hour cycles. The increase in dry weight was 3.5 times as great under the 6-second cycles as under the 30-minute cycles.

Effects of sucrose, IAA, and TIBA on growth with various cycles

Sucrose applied daily in the form of a 10 per cent spray to the leaves of cockleburs and tomatoes did not affect the growth patterns under the various light—dark cycles, as compared with the controls (Figures 1 and 3). The applications did increase the growth of cocklebur plants somewhat over that of the controls with all the cycles, however, the dry weight increase being 1.34 times more with sugar than without in the 2-hour cycle (Figure 1). Tomato growth was slightly decreased by the sugar spray with the four cycles employed (Figure 3).

The application of IAA also apparently did not affect the growth patterns significantly with either plant (Figures 1 and 3), although with some cycles the cocklebur growth was decreased somewhat and in one case was increased to 1.17 times that of the control (2-hour cycle). With the tomato growth was decreased to a considerable extent by the IAA application in all of the
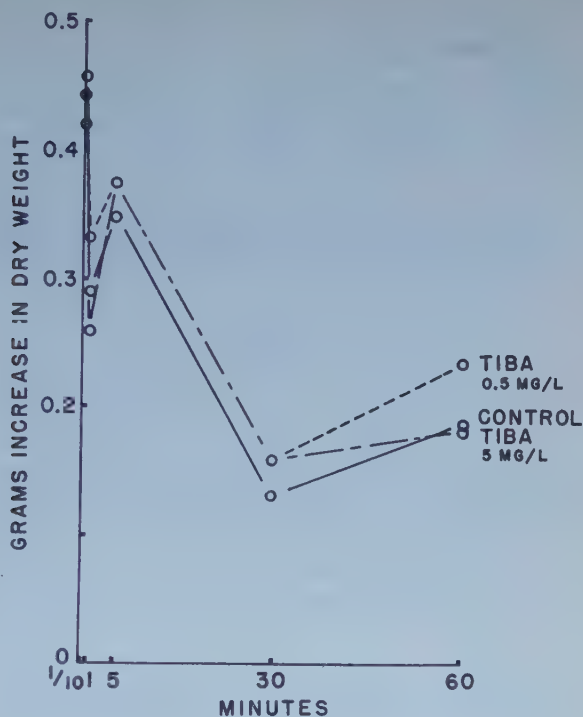
Figure 4. *Average increase in dry weight in 12 days of tops of tomato plants grown under various cycles of light and darkness. TIBA was applied as a spray at the beginning of the experimental period.*

four cycles employed as compared with the control. Somewhat of a change in the growth pattern appeared here also, in that growth in the 5-minute and 30-minute cycles was depressed more than in the longer 1-hour and 2-hour cycles as compared to the unsprayed control. This greater depressing effect in the two shorter cycles was also reflected in the fresh weight determinations, though not in the height increases with the various cycles. In the 2-hour cycle the dry weight increase of the control was 1.15 times that of the IAA-sprayed plants, while with the 5-minute cycle it was 1.58 times as great.

Application of the antiauxin TIBA to the leaves of the cockleburs (Figure 2) in a concentration of 5 mg/l did not alter the growth pattern with the various cycles used and did not affect the increase in dry weight particularly, except to increase it slightly in the 6-second, 5-minute, 30-minute, and 1-hour cycles, and to decrease it slightly in the 1-minute cycle. The tomato (Figure 4) also did not show any marked change of growth pattern with TIBA in 0.5 mg/l and 5.0 mg/l concentrations except to decrease or increase the dry weight gain slightly as compared to that of the unsprayed controls. In the 1-hour cycle the plants sprayed with 0.5 mg/l TIBA increased in dry weight 1.23 times as much as the control, but this was the greatest deviation.

Figure 5. *Effects of various cycles of light and darkness on growth of tomato plants in 12 days.* Top row: control; middle row: sprayed with 0.5 mg/l TIBA; bottom row: sprayed with 5.0 mg/l TIBA. Each plant is of the average height of a group of 9 given the same treatment.

Figure 5 is a photograph of the plants of Figure 4 and shows the typical responses of the tomato plants to a series of cycles.

Effects of sprays on chlorophyll formation

As reported in the previous paper, cocklebur plants grown with 5-minute and 15-minute cycles became light-green in color and the leaves contained less chlorophyll than the leaves of plants grown under longer cycles. The effects on chlorophyll were equally noticeable under all the 3 light intensities employed. In the series of cocklebur plants shown in Figure 2, the 5-minute and 30-minute plants were similarly light-green in color, whereas the 6-second and the 1-minute plants were dark-green and the 1-hour plants were intermediate in color.

With the tomato in the series of cycles illustrated in Figure 3 (5-minute, 30-minute, 1-hour, and 2-hour), the leaves in the two shorter cycles were also light-green in color, and the ones in the two longer cycles were of the normal dark color. In neither of the above series did the application of sucrose, IAA, or TIBA affect the chlorophyll deficiencies in the leaves.
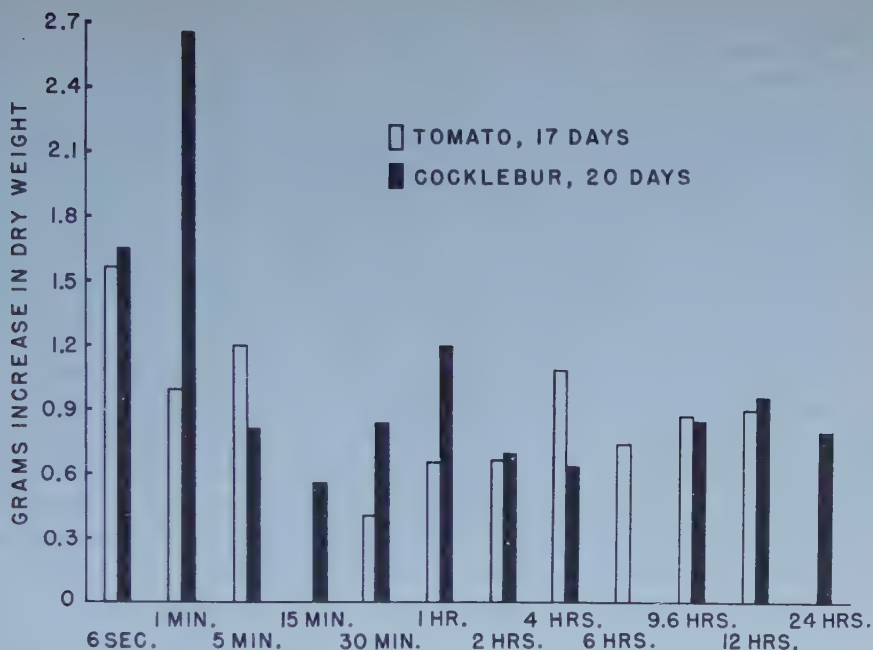
Figure 6. *Average increase in dry weight of tops of tomato and cocklebur plants grown under various cycles of light and darkness at 1000 foot-candles.* Data for this figure were derived from experimental periods of different lengths and were converted to a common length with the assumption that growth was proportional to time in the short periods concerned. The cocklebur was not tested under the 6-hour cycles and the tomato not under the 15-minute cycles.

Discussion

The data for some of the longer cycles from the previous paper have been combined with the data for shorter cycles secured in the present work in Figure 6 by converting to a growing period of the same length for each series of cycles tested, assuming for this purpose that the increases in dry weight were proportional to time. It can thus be seen that, starting with plants several weeks old and using relatively short experimental periods compared with the 5 to 6 weeks employed by Garner and Allard (3), the maximum growth in the cocklebur with the particular cycles employed took place in the 1-minute cycle, with considerably less growth in the longer cycles and somewhat less in the shorter 6-second cycle. A secondary maximum appears in the 1-hour cycle, with a third smaller peak in the 12-hour cycle. This pattern does not compare well with that found by Garner and Allard for the short-day Cosmos, where by far the greatest growth took place under 12-hour

"control" cycles and second-best growth under 5-second cycles, with a mini-
mum increase in dry weight under 1-minute cycles.

With the tomato in the present study the maximum growth took place
under the shortest cycles employed (6-second cycles), with a minimum under
the 30-minute cycle (a 15-minute cycle was not tested with the tomato). A
second maximum appeared with the 4-hour cycle, and a third smaller peak
with the more natural 12-hour cycle. These growth variations for the day-
neutral tomato correspond quite closely to those found by Garner and Allard
for the day-neutral buckwheat, in cases where the same cycles were em-
ployed.

As was pointed out in the previous paper, the possibility that inhibition of
growth under certain cycles of light and darkness is due to effects on the
photosynthetic mechanism does not seem very likely in that all of the plants
under the various cycles received the same total amount of light during the
experimental period. Furthermore, the daily application of sucrose sprays
which might be expected to offset photosynthetic upsets under certain of
the cycles did not alter the growth pattern for either plant.

Attempts to change the growth patterns by applications of IAA and TIBA
were unsuccessful. Neither IAA nor TIBA in the concentrations employed
affected the relative amounts of growth to any great extent, although, as
seen in Figure 1, IAA somewhat increased the growth in the 2-hour cycles
over that of the control in the cocklebur; and in Figure 3 it may be seen
that in the shorter 5-minute and 30-minute cycles the growth rate of the
tomato with IAA was depressed as compared to that with sucrose and with
the control. The fact that no clear-cut changes in the growth patterns were
produced by the applied auxin and anti-auxin indicates that there was
neither a pronounced decrease nor increase in auxin levels in the plants under
the influence of the various treatments. Presumably, then, the observed
growth differences were not due to deficiencies nor excesses of auxin. Since
auxin differences would first of all be reflected in differences in vegetative
growth, these experiments suggest that the influence of various light and
dark cycles on growth of the cocklebur and tomato is not mediated by auxin.

The results with the cocklebur in the present work indicate that over a
short period of time, at least, growth is favored by the 1-minute, 1-hour, and
12-hour alternations of light and darkness and inhibited by cycles in-between
in length. With the tomato growth is favored by the 6-second, 5-minute,
4-hour, and 12-hour alternations of the ones employed in this study. These
effects are difficult to explain on the assumption that a daily rhythm exists
in plant processes concerned with growth, as has been suggested by Bün-
ning (2) in connection with the responses of plants to environmental factors.

Summary

1. Young cocklebur plants grown under various short cycles of alternating light and dark periods of equal length (each period 6 seconds, 1 minute, 5 minutes, 30 minutes, 60 minutes, or 120 minutes in length) for two to three weeks showed the best growth as measured by increase in dry weight under cycles of 1 minute of light and 1 minute of darkness (1-minute cycles). One-hour cycles were also favorable to growth, while growth was relatively poor under 5-minute, 30-minute, and 2-hour cycles.

2. Young tomato plants grew best under the shortest cycle employed (6-second) and also well under 5-minute cycles, but growth was relatively poor under 1-minute cycles and quite poor under 30-minute and 60-minute cycles of the ones employed.

3. The growth patterns under a series of various cycles were not changed by the application of a daily spray of 10 per cent sucrose solution, by a spray of 5×10^{-4} M solution of IAA applied every third day, nor by a single spray of 0.5 or 5.0 mg/l of TIBA applied at the beginning of the experimental period in either plant, except for some depression of growth in the tomato by the applied IAA.

4. Chlorophyll deficiencies appeared in leaves of cocklebur plants grown under 5-minute and 15-minute cycles and in the leaves of tomato plants grown under 5-minute and 30-minute cycles (a 15-minute cycle was not used with the tomato), while plants under the other cycles used were normal in color. The application of the various sprays did not affect this chlorophyll deficiency.

References

1. Bonde, Erik K.: The effect of various cycles of light and darkness on the growth of tomato and cocklebur plants. — Physiol. Plant. 8: 913. 1955.
2. Bünning, E.: Ueber die photophile und skotophile Phase der endogenen Tagesrhythmik. — Planta 38: 521. 1950.
3. Garner, W. W. & Allard, H. A.: Effect of abnormally long and short alternations of light and darkness on growth and development of plants. — Journ. Agr. Res. 42: 629. 1931.

Studies on Polarity and Auxin Transport in Plants [1,2]

I. Modification of Polarity and Auxin Transport by Triiodobenzoic Acid

By

ETHEL NIEDERGANG-KAMIEN [3] and FOLKE SKOOG

Department of Botany, University of Wisconsin, Madison 6, Wisconsin
(Received October 28, 1955)

Introduction

It is well known that the distribution of auxin in plants involves a specific, polar transport mechanism (Went 1928; van der Weij 1932). It follows that phenomena of growth and morphogenesis regulated by auxin and sensitive to changes in hormone content will be modified by factors which influence the polarity and auxin transport. This holds whether or not these factors also influence growth in other ways.

In the past few years, several investigators have reported that treatment with 2,3,5-triiodobenzoic acid (TIBA) produces aberrant development in plants. e.g. loss of apical dominance, fasciation, formative effects on leaves, initiation and enhancement of flowering. In the proposed explanations of these effects the assumption has been generally made that TIBA lowers the effective auxin level in the tissues either by 'inactivating' the endogenous auxin or by competing with the auxin in the growth reaction (i.e., in some chemical reaction in which auxin participates).

Observations in this laboratory have suggested, however, that TIBA in low concentrations primarily affects the polarity of the tissues rather than their capacity to grow. Indeed, it appears that correlative phenomena are involved and that the effect of TIBA on the polar transport of auxin permits a satisfactory explanation of the striking morphogenic effects of this substance (9). The same conclusion has now been reached by Kuse (6).

Methods and Materials

The effect of TIBA on morphological polarity has been determined in terms of callus and organ formation in tobacco stem segment cultures. Its effects on polar movement and distribution of endogenous auxin or added indole-3-acetic acid (IAA) have been determined by transport experiments performed on sunflower and tobacco stem internodes.

Tissue cultures: — Tobacco plants, *Nicotiana tabacum*, variety Wisconsin No 38, were grown in the greenhouse to about three feet in height, harvested, and prepared for tissue culture as described by Miller and Skoog (8). After the outer tissues down to the cambium had been stripped away, *cylinders* approximately 1 cm. long were cut from the internodes. The cylinders were planted with either the morphologically basal or apical end in contact with the medium. (The terms apical and basal refer to the morphological apex and base of the plant regardless of the orientation of the stem cylinders or segments with respect to gravity.) In some experiments, each cylinder was divided into three *segments* by longitudinal cuts in a tangential plane and these segments were planted with the pith side down on the surface of the medium. White's nutrient solution with 1 per cent agar was used as control culture medium. Substances to be tested were added and the pH was adjusted to 4.5, prior to autoclaving. After autoclaving, the pH of the media was ca. 6. The cultures were grown in cabinets at room temperature and exposed to weak diffuse light for 6—7 weeks at which time observations were made. In recording the distribution of buds each cylinder was divided into two equal regions: the apical half and basal half. Determinations of weights were based on 8 –10 replicates. Fresh weights were determined as quickly as possible after harvesting and dry weights after the tissues had been dried to constant weight for 24—48 hours at 65° C. in a forced air oven.

Distribution of auxin (and added IAA) in tobacco stem cylinders: — The tobacco plants used consisted of ca. 50 cm. long stems with two young fully-expanded leaves left attached; the terminal portion of the shoot above these leaves and all other leaves had been removed. Sets of 7—8 plants were placed in 4 liter pyrex beakers with 1 liter of water or solutions of the chemical to be tested. To facilitate uptake of the solution the stems were severed ca. 4 cm. from the basal ends by an oblique cut performed below the liquid surface. Approximately 5 cm. of the stems were submerged. The plants were kept on a greenhouse bench and were exposed to the air current from a fan for a 4-hour period. At the end of this absorption period the stems were cut above the liquid surface and the lower portions discarded to avoid mechanical carry-over of chemicals. All subsequent operations were carried out in the Avena room at ca. 24° C. and 88 per cent relative humidity. The outer tissues

were stripped away and 1 cm. cylinders were excised with a double-bladed cutter. Between 30 and 40 cylinders, taken from the region of 15—25 cm. below the growing point, were prepared for each treatment. The cylinders were placed horizontally across glass rods in a moist chamber for a 2—3 hour period. They were then cut transversely into apical and basal halves which were dropped immediately into 50 ml. peroxide-free ether in 125 ml. Erlenmeyer flasks for auxin extractions. The flasks were quickly removed to a refrigerator at 4° C. for a total 2.5 hour extraction period. This short-time ether extraction presumably yields most of the so-called "free" auxin. The ether was decanted into test tubes, evaporated over a water bath, and the dry residue stored overnight in a refrigerator. The extracts were dissolved in warm water, and agar added to a final concentration of 1.5 per cent, cast into blocks, and tested for activity by the standard Avena test. Several dilutions were used, or pilot tests were run, so that the activity of the samples was measured at concentrations producing curvatures of less than maximum angles.

Assay methods: — The Avena tests were carried out in a dark room at a temperature of 24° C, humidity of 87—90 per cent, and illuminated occasionally with red light. Three-day-old Avena coleoptiles were decapitated with a razor blade and again decapitated three hours later by sharply breaking off the upper 3 mm. of the coleoptile with fine-pointed forceps. Agar blocks were applied and shadowgraphs made after 110-minute intervals. An activity curve for 0, 10, 20, 40, and 80 µg/l. IAA was prepared for most of the experiments and for the others a reference IAA concentration of 20 µg/l. was used. The curvatures obtained with the tissue extracts were evaluated in terms of IAA equivalents from the standard curve.

For colorimetric assays of IAA, Gordon and Weber's modified Salkowski test was used (3). Readings were made at 450 mµ with a Beckman DU Spectrophotometer. Ascending paper chromatograms were made with Whatman No. 1 filter paper and an isobutanol-water-30 per cent ammonia solvent system (10:1:1).

Auxin transport through sunflower stem segments: — Stem cylinders, 0.5 cm. in length, were removed with a double-bladed cutter from the internode above the cotyledons of three-week-old sunflowers. These were stood, basal end down, on moist filter paper for an hour to allow the endogenous 'free' auxin to move out. The stem cylinders were placed in holders so as to keep the identity of the apical and basal ends, and soaked just beneath the surface for 2 hours in distilled water or in the desired concentrations of the compounds to be tested. Six stem cylinders were placed basal end down on an agar block 12 times standard size (10 mm.³), and another agar block containing 2 mg./l. IAA was placed on top. The cylinders had been carefully blotted dry to prevent leakage of IAA along the surfaces and the cut ends moistened with a small drop of distilled water to insure good contact with the agar blocks. Duplicate sets were made for each treatment and placed in a moist chamber in the Avena room for transport periods of 2 or 2.5 hours. The recipient agar blocks, 6 from each duplicate, were tested for activity by the standard Avena test. The amount of IAA transported acropetally through the cylinders was determined with the cylinders placed in an inverse position. The inactivation occurring in the receptor blocks was estimated by determining the loss in activity in agar blocks with known IAA concentrations which had been placed in contact with cut basal ends of control and treated stem cylinders during the transport period. The observed transport values were then corrected by the amount of this apparent "loss".
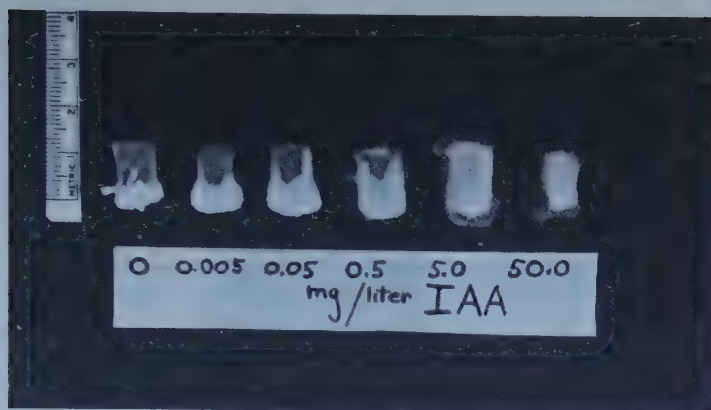
Figure 1. *Effect of IAA on the distribution of callus in tobacco stem segments cultured with the entire pith surface in contact with the media.* Age of cultures ca. 30 days.

Results

Morphological polarity in excised tobacco stem cultures: — In *segments* of tobacco stem, cultured on White's medium, callus is normally produced only at the basal end. Callus formation increases and spreads toward the apical ends of the segments as the IAA concentration in the medium is increased; with 5 mg./l. or higher concentrations of IAA, the growth of callus is evenly distributed over the entire surface (Figure 1). In *cylinders* of tobacco stem, planted with either the apical or basal end in contact with the medium, the callus is likewise restricted to the basal end. Also in the presence of IAA (in contrast with the behavior in the segments) the growth of callus occurs only at the basal end when the cylinders are planted with this end in contact with the medium. If, on the other hand, the apical end is placed in contact with the medium, callus is formed all over the sides of the cylinders (Figure 2). The difference in callus distribution in cylinders in the normal and inverse positions is due, no doubt, to the almost complete absence of a net acropetal transport of IAA. In general, therefore, with *mode· rate IAA supplies, the amount of callus growth is related to the total auxin content and the distribution of the callus is dependent on the auxin gradient within the stem segments or cylinders.*

Effect of TIBA on morphological polarity: — The addition of TIBA to the culture medium markedly affects the distribution of callus growth (Figure 3). With 10 mg./l. TIBA added, callus formation extends up to the apical end of the cylinders, although it is still greater at the basal end. With 50 mg./l. TIBA, it becomes uniformly distributed over the entire lateral
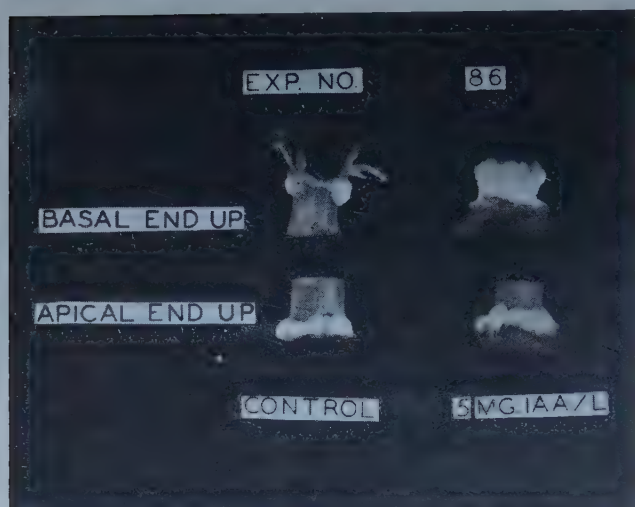
Figure 2. *Callus distribution on tobacco stem cylinders oriented basal or apical end up on control or IAA-containing media.* Age of cultures 36 days; started Jan. 10, 1954.

surface of the cylinders. The concentration at which uniform distribution is reached varies from one experiment to the next but is frequently as low as 20 mg./l. It should be noted that in the presence of TIBA — and whether or not IAA is present — uniform distribution of callus growth is obtained regardless of which end of the cylinder is placed in contact with the medium.

In stem segment cultures, in which the entire pith surfaces are in contact with the medium, 2.5 mg./l. TIBA generally suffices to produce an even distribution of callus (Figure 4).
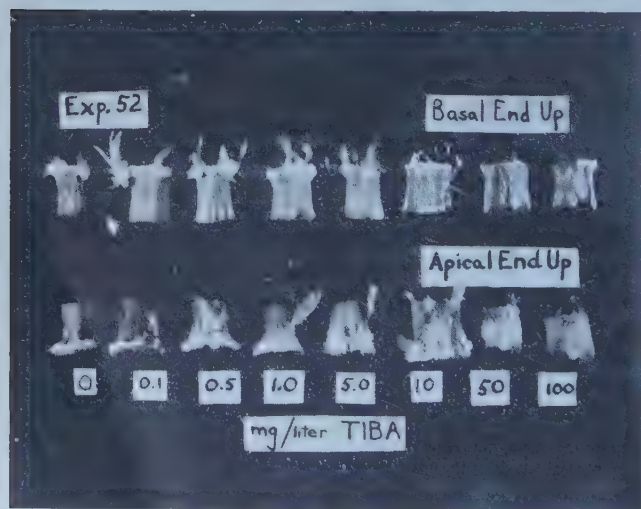
Figure 3. *Effect of TIBA on callus distribution on tobacco stem cylinders.* Age of cultures 25 days; started April 9, 1953.

Figure 4. *Effect of TIBA on callus distribution in tobacco stem segments cultured with the entire pith surface in contact with the media.* Age of cultures 49 days; started Mar. 7, 1952.
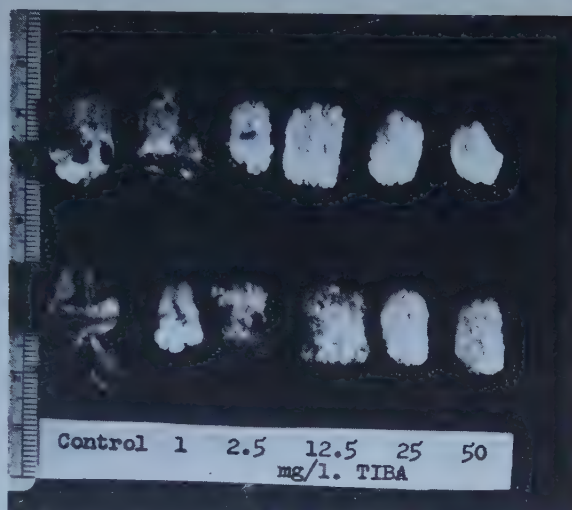
The effect of TIBA on the distribution of callus growth is not due to inherent growth promoting activity of the substance, for the total growth of callus is only slightly affected. In five experiments with serial concentrations of TIBA in the range from 0.50 mg./l. the average increase in dry weight over that of the control was only 13 per cent, whereas the optimal concentrations (1—10 mg./l.) of IAA, 2,4-dichlorophenoxyacetic acid, and naphthaleneacetic acid gave increases of 299, 127, and 420 per cent respectively. Much of this weight increase resulted from enlargement of pith cells at the surface in contact with the nutrient medium.

A marked relocation of organ regeneration, as well as the non-polar distribution of callus, reflects an effect of TIBA on polarity. Under the conditions of these experiments, buds usually are formed only at the basal end in control cylinders, but with increasing TIBA concentrations the region of most active bud formation shifts progressively toward the apical end (Figure 5).

Distribution of auxin in control and in TIBA-treated tissues: — The distribution of the endogenous ("free") auxin in the control tobacco stem cylinders (Table 1) changes rapidly from an even 50/50 distribution immediately after they are prepared (column 3) to about 15 per cent in the apical halves and 85 per cent in the basal halves within a three hour period (column 6). IAA supplied to the tissues becomes similarly unequally distributed (column 9) and presumably endogenous auxin as it is released from the 'bound' form would likewise accumulate at the basal end.

The effect of TIBA on the endogenous auxin content as determined by

5
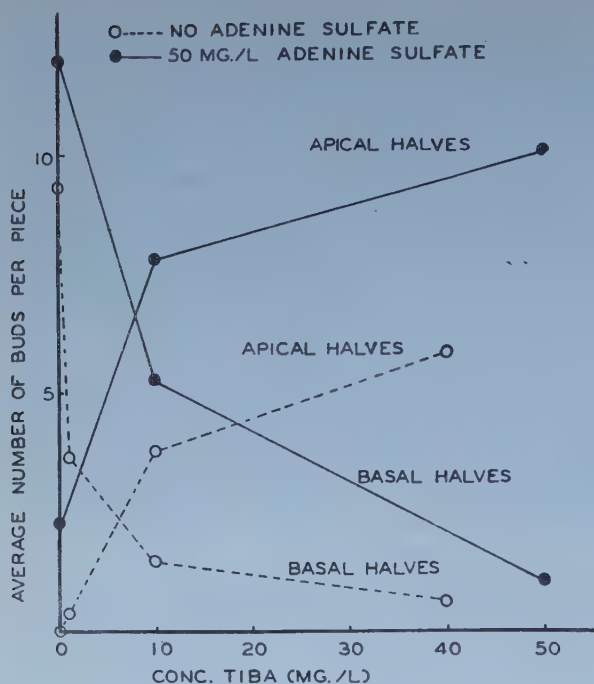
Figure 5. *Bud distribution in tobacco stem cylinders cultured basal end down on nutrient medium with increasing concentrations of TIBA. The shift in distribution is the more evident with the increased number of buds in presence of adenine sulfate. Age of cultures with AdSO₄, 72 days, started Dec. 17, 1952; and without AdSO₄, 61 days, started Oct. 8, 1953.*

ether extraction is two-fold (Table 2). The difference in auxin content between the apical and basal halves is markedly reduced and sometimes disappears completely (column 3); and the total amount extractable is lower (down from 0.21 to 0.07 µg./gm. dry wt.). It is difficult, therefore, to decide whether the equal distribution of auxin in the two halves has resulted from an inhibition of polar transport, from loss in activity, or from the retention of material in the tissue. The lower auxin level might also be a result of less transport from centers of production or from an exogenous IAA source in the stem during the absorption period prior to the preparation of the segments for analysis. To clarify these points, IAA was introduced into the stem, during the last two hours of the 4-hour absorption period in which TIBA was administered as described above. This raised the level of extractable auxin in the tissue from an average of 0.21 to 2.38 µg/gm. dry wt. in the absence of TIBA (Table 1), and the distribution of this auxin was polar in the same manner as that of the endogenous auxin. In the TIBA treated stems the level of extractable auxin was also greatly increased (from 0.07 to 1.08 µg/gm. dry wt.) but, as expected, was lower than in the IAA treated controls. There was still the same marked effect of TIBA on the polar transport; the auxin contents of the two halves were nearly the same (Table 1, column 6). These results exclude auxin destruction as a factor in the distri-

Table 1. *Distribution of endogenous auxin or added IAA in tobacco stem cylinders from control plants.* Activity determined by Avena tests of ether extracts obtained in 2.5 hours at 4° C. Values reported as µg IAA equivalents/gm. dry weight.

| Exp. No. | Immediately After Cutting | | | 3 Hours After Cutting | | | | | |
| | Endogenous Auxin | | | Endogenous Auxin | | | Plants Previously Supplied with 10 mg./l. Indoleacetic Acid | | |
	(1) Apical Halves	(2) Basal Halves	(3) Ratio Apical/Basal	(4) Apical Halves	(5) Basal Halves	(6) Ratio Apical/Basal	(7) Apical Halves	(8) Basal Halves	(9) Ratio Apical/Basal
55	0.42	0.45	48/52	0.0	0.30	0/100			
56	0.30	0.32	48/52	0.15	0.40	27/73			
76				0.01	0.16	6/94			
80				0.01	0.09	10/90			
84				0.01	0.09	10/90			
92				0.01	0.06	14/86	0.21	0.96	18/82
101							0.31	2.73	10/90
102							0.32	2.61	11/89
Avg.	0.36	0.39	48/52	0.03	0.18	11/89	0.28	2.10	13/87
Avg. Total	0.75			0.21			2.38		

bution of activity in the cylinders, because the content in the apical halves of the TIBA treated cylinders (Table 2, column 4) was now greater than in the controls (Table 1, column 7). Thus, even if differential destruction occurs, it in itself would not account for the lack of polar distribution.

Table 2. *Effect of TIBA on the distribution of endogenous auxin or added IAA in tobacco stem cylinders.* Activity determined by Avena tests of ether extracts obtained in 2.5 hours at 4° C. Values reported as µg. IAA equivalents/gm. dry weight.

| Exp. No. | TIBA mg/l. | Auxin Contents — 3 Hours After Cutting | | | | | |
| | | Endogenous Auxin | | | Plants Previously Supplied with 10 mg/l. Indoleacetic Acid | | |
		(1) Apical Halves	(2) Basal Halves	(3) Ratio Apical/Basal	(4) Apical Halves	(5) Basal Halves	(6) Ratio Apical Basal
63	100	0.10	0.10	50/50			
80	10	0.02	0.04	33/67			
	25	0.01	0.01	50/50			
84	1	0.02	0.05	29/71			
92	20	0.004	0.009	31/69	0.42	0.49	46/54
101	20				0.27	0.27	50/50
102	20				0.66	1.08	38/62
Avg.		0.03	0.04	39/61	0.45	0.61	45/55
Avg. Total		0.07			1.06		
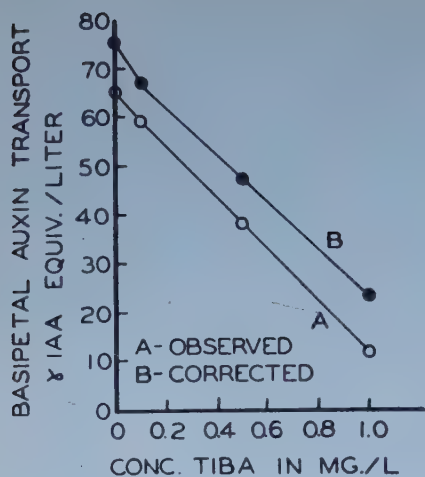
Figure 6. *Effect of TIBA concentration on auxin transport in 21-day old sunflower epicotyl cylinders.* Length of cylinders 5 mm. Duration of pretreatment with TIBA 2 1/2 hours. Transport time, 2 1/2 hours.

To investigate further this difference in activity level between the control and TIBA-treated tissues, ether extracts prepared by the identical method as those assayed by the Avena test, were analyzed by the Salkowski test. It was first established that TIBA did not interfere with this reaction. The amounts of auxin in the extracts from the control tissue, as determined spectrophotometrically, were of the order of 2 µg/gm. dry weight, in good agreement with the values obtained by the bio-assay (average of 2.4 µg/gm. dry wt.). It is of interest that with these auxin extracts the Salkowski reagent did not give the violet color typical of IAA, but rather a transitory blue. However, after autoclaving at an alkaline pH, at 15 lbs. for 10 minutes, prior to testing with the Salkowski reagent, the violet color did appear. This may indicate that IAA, added to plant tissues, and perhaps also the endogenous auxin, is transported in some form other than free IAA. With the TIBA treated tissues, there was little or no color formation either before or after the alkaline treatment. On chromatograms, the color was violet: thus it is possible that the presence of NH_4OH in the solvent during development of the chromatograms may have "hydrolyzed" the extracts. A chromatogram of the extracts with isobutanol-water-ammonia as the solvent system gave the following results:

Reference Spot:	Relative intensity of color	R_f
IAA-10 µg	+++++	.47
Extracts:		
H₂O-Apical	+	.47
H₂O-Basal ......:....	+++	.47
TIBA-Apical	+	.47
TIBA-Basal	+	.47

Table 3. *Effect of pretreatment with TIBA on the transport of IAA through sunflower epicotyl cylinders.* Data expressed as µg-IAA equivalents/l. Plants used when epicotyls were 4 cm long, age 21 ± 3 days. Pretreatment by soaking in H_2O or TIBA solutions.

Exp. No.	Conc. TIBA mg/l.	Time in hrs.		µg-IAA equivalents/l. transported					
		Pretreatment	Transport	Controls			TIBA-treated		
				Obsd.	Loss	Total	Obsd.	Loss	Total
34	1	2	3.0	6.7	17.0	23.7	0.0	20.0	20.0
38	1	2	3.0	21.3	16.6	37.9	0.0	20.1	20.1
59	1	3	1.5	67.2	19.1	86.3	17.3	20.0	37.3
87	1	2.7	2.5	36.7	4.5	41.2	10.0	6.5	16.5
103	20	2.5	2.0	19.6	6.2	25.8	1.0	8.7	9.7
Average				30.3	12.7	43.0	5.7	15.1	20.8

Possibly the TIBA treatment results in some loss or "fixation" of ether extractable endogenous auxin or added IAA or both in the tobacco tissues.

Effect of TIBA on auxin transport through tissue sections: — That TIBA affects the polar auxin-transport mechanism is borne out by experiments with sunflower stem cylinders pretreated for 2—3 hours with TIBA solutions. The results of one experiment are shown in Figure 6 and the results of all experiments are summarized in Table 3. The quantity of IAA transported is seen to decrease markedly and in proportion to the TIBA concentration in the preteatment solutions. For example, the 1mg./l. TIBA pretreatment decreased the IAA transport by the amount 53.8 µg/l., or, as shown by suitable controls, 42.4 µg/l. more than could be accounted for merely through loss in activity in the receptor blocks. In fact, in no case could the differences between the transport of IAA in the control and treated tissues be explained on the basis of IAA inactivation in the receptor blocks in contact with the tissue surfaces. In transport experiments with 5 mm. Avena coleoptile cylinders a similar interference of TIBA with IAA transport was demonstrated.

The type of curvature obtained with IAA in Avena tests with coleoptiles pretreated with TIBA gives a further striking indication that transport of IAA was inhibited. TIBA itself produces no curvatures indicative of activity, but concentrations above 1 mg./l. give positive curvatures indicative of growth inhibition (2, 12, 13). If agar blocks with 5 mg./l. TIBA are applied to the cut surface of the coleoptiles for 1 hour as a pretreatment and 20 µg./l. IAA blocks are then tested, the resulting curvatures are restricted to the uppermost 2—3 mm. of the coleoptile. This type of curvature has been demonstrated with growth promoting compounds which are not transported readily, such as indeneacetic-, phenylacetic-, or 2,4-dichlorophenoxyacetic acid, and indicates that the pretreatment of the coleoptiles with TIBA

inhibits the polar transport of IAA while still permitting at least a partial expression of its "growth" activity.

Discussion

Tobacco stem cylinder cultures have been found to be particularly suitable for investigations of polarity. The cylinders consist of differentiated cambial, xylem, internal phloem, and pith tissue with no apparent morphological differentiation of apical and basal halves at the beginning of the experiments. They are easy to maintain for periods of several weeks under relatively constant conditions and will grow to some extent on their endogenous growth factor reserves. They show marked responses in growth and differentiation to IAA and to other growth factors. Furthermore, as shown here, these tissues respond to certain chemical treatments by changes in amount and distribution of callus formation, pith enlargement, regeneration of buds and roots, modification in leaf form, etc. As the control cylinders exhibit a strictly polar pattern of growth and differentiation, any treatment which modifies polarity is readily apparent.

Such changes when they have been confirmed by standard determinations of auxin distribution, by extraction techniques, and transport experiments, are highly sensitive tools which can be conveniently used in studies of polarity.

It is concluded, therefore, that the principal influence of low TIBA concentrations is on the polarity of auxin transport, and that many of the physiological effects of the substance can be readily explained on this basis.

Certain effects of TIBA on correlative phenomena are of special interest in this connection, as for example:

1. Snyder (11) found that 2 per cent TIBA applied in lanolin as a ring around the stem of Red Kidney beans could release the inhibition of axillary buds due to endogenous auxin or applied indolebutyric acid. Similarly, we have removed, in beans, the inhibition of the lateral buds by application of 1 per cent TIBA in lanolin as a ring around the stem between the auxin source and the lateral bud, the auxin source being either the terminal bud or 1 per cent IAA in lanolin.

2. In experiments with short stem sections of sweet potato, Kuse (6) demonstrated that TIBA applied in a lanolin ring to the petiole of a leaf prevented the inhibition of the lateral bud by either the leaf blade or IAA in lanolin substituted for the leaf blade. The treatment was effective only if the TIBA was applied between the point of application of the IAA and the lateral bud. He was also able to prevent the transmission of the photo- or geo-tropic stimuli by TIBA treatment.

Also the following phenomena might be interpreted as resulting from altera-tions in the local concentration levels of auxin because of the action of TIBA on the polar distribution of the hormone.

Treatments with TIBA which have been reported to

(a) increase the number of basal shoots in pruned rose bushes (11).
(b) increase tillering in grasses (7).
(c) increase the number of flowers in soybeans (2).
(d) cause premature flower bud initiation in tomato seedlings (14).
(e) cause "formative effects" suggestive of disturbances in correlation between parts in leaves (2, 4, 5, 10, 11, 17, 18).

Although TIBA has been investigated most intensively, results of experi-ments with various other growth substances, indicate that many of these also affect polar auxin transport. It would appear that compounds now classified loosely as weak auxins, anti-auxins, auxin antagonists, etc. might well be examined for their possible influences on the polar auxin transport mecha-nism. For example, it has been found that phenylacetic-, 2,4-dichlorophen-oxyacetic- and phenylbutyric acids affect polar auxin transport to some extent, whereas transcinnamic acid does not. The fact that 2,4-dinitrophenol, which interferes with phosphate energy utilization, also interferes with polar auxin transport is interesting in that it may be considered as a distant rela-tive of TIBA and thus may give a clue to the mode of action of these substances as a group in their disruption of polar transport. On the other hand, growth inhibiting concentrations of maleic hydrazide are inactive in these tests, thus indicating a sharp distinction between the structural pro-perties of auxin required for growth and for polar transport.

Summary

Treatment of plants with TIBA produced marked changes in polarity which were evident in the following ways:

In tobacco stem cylinders or segments, cultured on modified White's medium with added TIBA, callus formation occurred uniformly on the entire lateral surfaces and bud formation occurred at the apical end, whereas both occurred strictly at the basal end in control pieces.

The interference of TIBA with polar auxin transport was demonstrated in several ways:

First by direct analysis of stem segments or cylinders, i.e. by comparisons of the apex/base distribution of endogenous auxin and of added IAA previ-ously absorbed by the plants which had been treated with water or TIBA

solutions. In the control stem segments or cylinders the auxin contents in ether extracts of the apical and basal halves respectively, as measured either by Avena tests or colorometrically with a modified Salkowski test, showed a shift from an initial 50/50 to about a 15/85 apex/base distribution within 2 to 3 hours. In segments or cylinders from plants pretreated with TIBA the apex/base distribution remained close to 50/50, thus demonstrating that the polar transport had been stopped. This result was clear even though the total auxin contents, as expected, were lower in the TIBA treated plants.

Second, the transport of IAA through 5 mm. sunflower stem cylinders was shown to be prevented by TIBA pretreatment.

Third, IAA curvatures were shown to be localized to the upper 2 mm. in Avena coleoptiles which had been pretreated with TIBA. This restricted curvature is a characteristic response to nonpolarly transported auxins and thus is evidence of interference of TIBA with the transport of IAA.

The effect of TIBA on polarity of auxin transport is discussed in relation to reports in the literature of marked influences of this compound in correlative growth phenomena, and it is concluded that many physiological effects of TIBA on plants can be explained in terms of the observed loss of polar auxin transport.

References

1. Asen, E. & Hamner, C. L.: Effect of growth-regulating compounds on development of basal shoots of greenhouse roses. — Bot. Gaz. 115: 86. 1953.
2. Galston, A. W.: The effect of 2,3,5-triiodobenzoic acid on the growth and flowering of soybeans. — Am. J. Bot. 34: 356. 1947.
3. Gordon, S. A. & Weber, R.: Colorimetric estimation of indoleacetic acid. — Plant. Physiol. 26: 192. 1951.
4. Gorter, Chr. J.: The influence of 2,3,5-triiodobenzoic acid on the growing points of tomatoes. — Proc. Kon. Ned. Akad. Wetensch. Amsterdam 52: 1185. 1949.
5. Kraus, E. J. & Mitchell, J. W.: Growth-regulating substances as herbicides. — Bot. Gaz. 108: 301. 1947.
6. Kuse, G.: Effect of 2,3,5-triiodobenzoic acid on the growth of lateral bud and on tropism of petiole. — Memoirs Coll. Sci., Univ. Kyoto, Series B. 20: 3 Art. 12. 1953.
7. Leopold, A. C.: The control of tillering in grasses by auxin. — Am. J. Bot. 36: 6. 1949.
8. Miller, C. O. & Skoog, F.: Chemical control of bud formation in tobacco stem segments. — Am. J. Bot. 40: 10. 1953.
9. Niedergang, Ethel & Skoog, F.: AIBS Meetings, Cornell University, Ithaca, N.Y. September 1952.
10. Resende, F. & Viana, M. J.: Some effects of synthetic auxins and anti-auxins on the floral state. — Bol. Soc. Portug. Ci. Nat. 4 (2 a S): 74. 1952.
11. Snyder, W. E.: Some responses of plants to 2,3,5-triiodobenzoic acid. — Plant. Physiol. 24: 195. 1949.
12. Thimann, K. V. & Bonner, W. D.: The action of triiodobenzoic acid on growth. — Plant. Physiol. 23: 150. 1948.

13. de Waard, J. & Florschutz, P. A.: On the interaction of 2,3,5-iodobenzoic acid and indole-3-acetic acid in growth processes. — Proc. Acad. Sci. (Amsterdam) 51: 1317. 1948.
14. — & Roodenburg, J. W. M.: Premature flower-bud initiation in tomato seedlings caused by 2,3,5-triiodobenzoic acid. — Proc. Acad. Sci. (Amster.) 51: 248. 1948.
15. van der Weij, H. G.: Der Mechanismus des Wuchsstofftransportes I. — Rec. trav. bot. neerl. 29: 381. 1932.
16. Went, F. W.: Wuchstoff und Wachstum. — Rec. trav. bot. neerl. 25: 1. 1928.
17. van Zeist, W. & Kuevoets, Th. C. M.: Kalanchoë Blossfeldiana treated with 2.3.5-triiodobenzoic acid. — Proc. Acad. Sci. Amsterdam 54: 126. 1951.
18. Zimmerman, P. W. & Hitchcock, A. E.: Flowering habit and correlation of organs modified by triiodobenzoic acid. — Contrib. Boyce Thomp. Inst. 12: 491. 1942.

The Growth-Stimulating Effect of Fructose-1,6-Diphosphate on Boletus variegatus and Collybia velutipes

By

Harald Nilsson

Institute of Physiological Botany, University of Uppsala
(Received November 2, 1955)

Introduction

During the research on the metabolic requirements of different Hymeno-mycetes it has been shown repeatedly that unidentified substrates exert growth-stimulating effects that can not be accounted for in terms of tested substances. To mention some, Fries (1938) reported the stimulating influence of malt extract and yeast extract on wood-destroying Hymenomycetes, where at least the effect of the yeast extract could not be accounted for by com-paring with thiamine. He has also described positive effects on the spore germination (Fries 1943) of some soil-inhabiting Hymenomycetes of the action of malt extract and living colonies of other fungi. Melin (1946) has shown that water extracts of litter of forest trees have a fairly general growth-promoting effect on some mycorrhiza-forming Hymenomycetes and Gastero-mycetes, and Melin and Norkrans (1948) demonstrated the positive influence of water extracts from sporophores of various Hymenomycetes. It was concluded that this last effect was wholly or partly caused by amino acids in the extract from the sporophores. As to the influence of unidentified substrates the positive effect of some living organisms on others may be noted. Here will be mentioned only Melins dicovery of the strongly stimu-lating effect of pine and tomato roots on a large group of tested Hymeno-mycetes (Melin 1953, 1954; Melin and Das 1954). In this connection he concludes (Melin 1953) that the growth promoting metabolites can not be

included among known B-vitamins, purine and pyrimidin bases, or the amino acids of casein hydrolysate.

Through the discovery of the prominent rôle of the phosphate ester bond of various organic compounds in the energy transformations of the cell a new group of compounds of high interest has become known, and many of them are now available for physiological tests. It therefore seemed desirable to study the effect of some phosphorylated compounds on the growth of some Hymenomycetes. This paper reports some preliminary experiments with fructose-1,6-disphosphate.

Material and Method

Material. One of the tested fungi, *Boletus variegatus* (Sw.) Fr. is a mycorrhiza former with pine, while the other, *Collybia velutipes* (Curt. ex. Fr.) Quel. is purely saprophytic. Of *C. velutipes* two strains, L 1 and L 7, were tested. Dr. Karin Aschan, who kindly supplied these two strains to the author, has also given a good description of them (Aschan 1952). *C. velutipes*, L 7, as well as *B. variegatus* grows with fairly well developed aerial hypae, while *C. velutipes*, L 1, has a sparse development of these. Both fungi grow well on a liquid substrate of salts, glucose and thiamine.

Method. The pure cultur technique used was the following:

In 125 ml Erlenmeyer flasks of Pyrex glass 10 ml of a double-concentrated nutrient solution was sterilized by autoclaving at 120° C and 1.2 atm. The basic nutrient solution had the following composition:

Glucose	20.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
NH_4-tartrate	0.5 g
Fe-citrate (1 % soln.)	0.5 ml
$ZnSO_4$ (Zn-conc. $1/_{500}$)	0.5 ml
H_2O-redistilled 1000	ml

This solution was supplemented with a mixture of B-vitamins and the amino acids of casein. The sterilized flasks were thus filled aseptically with 1 ml/flask of a vitamin mixture containing (Norkrans 1950):

Thiamine	1 µg	Pyridoxine	1	µg
Ca-pantothenate	1 µg	Riboflavin	1	µg
Folic acid	1 µg	Biotin	0.01	µg
Nicotinamide	1 µg	Choline	10	µg
p-Aminobenzoic acid	1 µg	Inositol	1	mg

and 1 ml/flask of an amino acid mixture solution holding 10 mg amino acids per ml and with the following composition (Schmidt 1944, Melin and Norkrans 1948):

Glycine	0.5 per cent	L(—)-Proline	9.1 per cent
DL-Alanine	1.9	L(—)-Hydroxyproline ;	0.2
DL-Valine	7.9	DL-Serine	0.5
L(—)-Leucine	4.9	DL-Threonine	3.5
DL-Icoleucine	4.9	L(—)-Cystine	0.3
DL-Phenylalanine	3.9	DL-Methionine	3.4
L(—)Tyrosine	6.6	L(+)-Arginine	3.8
L(—)-Tryptophan	2.2	L(—)-Histidine HCl	2.5
L(+)Glutamic acid	32.2	L(+)-Lysine (HCl)$_2$	6.0
L(+)Aspartic acid	4.1		

The fructose-1,6-diphosphate concentrations (calcium salt from Schwartz Laboratories Inc., New York) used in the different experiments were adjusted to 2 ml/flask and the total amount of liquid per flask was adjusted to 20 ml by adding 6 ml sterilized redistilled water.

The sugar phosphate solutions were sterilized by filtering through a bacterial filter (Jena, 3G5).

The inoculations were made with pieces of inocula (Fries 1941), the growth of which had been initiated by placing them on Hagem-agar plates (Norkrans 1950) 24 hours before the inoculation of the flasks. In one experiment the inoculations were made with a mycelium suspension (Wikén et.al. 1951) of which 1 ml was transferred to each flask.

The incubation temperature was 25° C.

The experimental results are expressed as relative values of dry weights. The determination of dry weights was made in the usual way by collecting the mycelia in a dense wire-netting, washing them with distilled water, drying them for some hours on a filter paper at room temperature and finally drying them for 20 hours in weighing bottles at 105° C. They were then allowed to cool in a desiccator before being weighed on an analytical balance. Each relative value is based on a mean weight value of 5 parallels.

Experiments and results

In a preliminary experiment the effect of several sugar phosphates was tested on Boletus variegatus. Besides fructose-1,6-diphosphate, glucose-1-phosphate (dipotassium salt), glucose-6-phosphate (barium salt), fructose-6-phosphate (barium salt) and a mixture of these were tested. They were given in two concentrations, viz. 0.1 per cent and 0.025 per cent with regard to the sugar unit they contained. The flasks were inoculated with pieces of inocula and the incubation time was 12 days.

Figure 1 shows the relative values of the growth at the lower concentration. The higher concentration inhibited growth almost completely. As can be seen from the figure the three monophosphates had no significant effect on the growth, whereas the diphosphate and the mixture showed a distinct
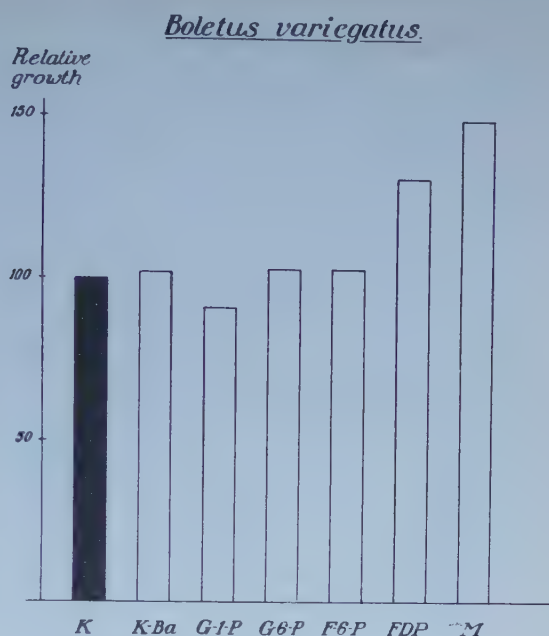
Figure 1. *Relative growth of Boletus variegatus on different sugar phosphates.* K=control; K-Ba=Barium control; G-1-P=glucose-1-phosphate; G-6-P=glucose-6-phosphate; F-6-P=fructose-6-phosphate; FDP=fructose-1,6-diphosphate; M=mixture. The concentration was 0.025 per cent with regard to the sugar unit.

positive effect under the given set of conditions. The effect of the mixture must, of course, be ascribed to the diphosphate. That the barium in glucose-6-phosphate and fructose-6-phosphate failed to influence the growth can be seen from the figure.

The conclusion drawn from the experiment was that fructose-1,6-diphosphate had a marked effect on Boletus variegatus, but that the concentration range must be lowered somewhat.

Consequently the next experiment was undertaken to study the effect of fructose-1,6 diphosphate at different concentrations. The following concentrations were tested: 0.05, 0.025, 0.013, and 0.0065 per cent with regard to the sugar unit in the fructose-1,6-diphosphate molecule. The flasks were inoculated with a mycelium suspension of Boletus variegatus and the cultural flasks were shaken in a shake-equipment during the time of incubation. The mycelia were harvested after 7 days.

The results can be seen in figure 2. It is quite obvious that the highest concentration (0.05 per cent) inhibited the growth, while the others stimulated it markedly. Of greatest interest is the fact that the lowest concentration had the highest stimulative effect. The mycelial production was almost twice that of the control.

The third experiment reported here was made with two strains of Collybia velutipes, L 1 and L 7. It aimed at finding out whether a purely saprophytic

Boletus variegatus.
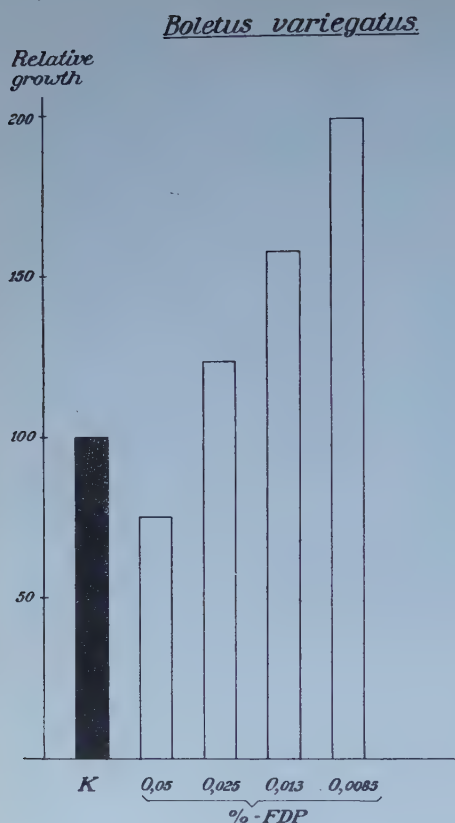
Figure 2. *Relative growth of Boletus variegatus at four different concentrations of fructose-1,6-diphosphate* (FDP). K means control.

species with a good growth and with no special metabolic requirements would respond to fructose-1,6-diphosphate. The two strains were tested at the three concentrations that had a positive effect on Boletus variegatus in the previous experiment, *viz.* 0.025, 0.013, and 0.0065 per cent. The inoculations were made with pieces of inocula and the time of incubation was 10 days.

The results are illustrated in figure 3.

It is quite evident that also the two strains L 1 and L 7 of Collybia velutipes respond positively to the fructose-1,6-diphosphate and that here, too, the effect on mycelial production is quite marked. However, the figures also reveal that the two strains do not respond in the same way. The growth response of strain L 7 is similar to that of Boletus variegatus with better growth at the lower concentrations of the diphosphate. As has been mentioned earlier, the appearance of the mycelial colonies with a good development of aerial-hyphae is the same. Conversely C. velutipes, L 1, responds with a better growth at the highest concentration of the sugar phosphate. Whether
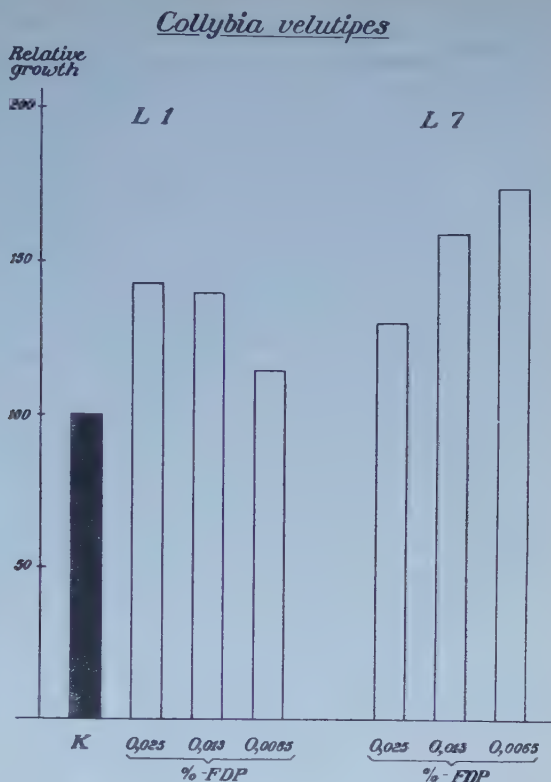
Figure 3. *Relative growth of two strains, L 1 and L 7 of Collybia velutipes at three concentrations of fructose-1,6-diphosphate* (FDP). K means control.

this has something to do with the more submersed development of its mycelial colonies is not easy to say, but the parallelism between the two phenomena is striking.

Discussion

From the experiments reported above it is quite clear that *fructose-1,6-diphosphate had a very striking growth-promoting effect on Boletus variegatus and on the two wild type strains L 1 and L 7 of Collybia velutipes.* It must also be stated that, at least for Boletus variegatus, the effect is the reverse above a certain concentration of the diphosphate. It is extremely probable that the same should apply to Collybia velutipes, even if the concentrations of inhibitions may not be the same as for Boletus variegatus.

Again the concentrations for growth-promotion reveal that the fructose-1,6-diphosphate cannot to any great extent be used as a carbon source. At this stage of investigation it is, however, too early to offer any satisfactory explanation of the phenomenon. It may be guessed that the effect is coupled

with the general role of the fructose-1,6-diphosphate in the energy releasing metabolism. But in this connection the attention should be drawn to the enlightening discovery by Leloir, *et al.* (1948) later confirmed by Sutherland *et al.* (1949) that the coenzyme for the phosphoglucomutase reaction is glucose-1,6-diphosphate itself. As it was also found by Leloir et al. (Leloir *et al.* 1948, Leloir 1951) that glucose-1.6-diphosphate was an impurity of the examined preparations of hexose phosphates, it can be suspected that the effects of fructose-1,6-diphosphate · recorded here might be due to impurities of glucose-1,6-diphosphate in the preparation used by the author. No investigation into this point has been made. On the other hand such an explanation seems incompatible with the fact that the 1-carbon phosphate group of glucose-1,6-diphosphate is acid labile and the experiment were performed in an acid medium (initial pH 5.2 and end pH 3.4—4.1). Nor did the product of the acid hydrolysis of glucose-1,6-diphosphate, *viz.* glucose-6-phosphate or a preparation of glucose-1-phosphate have any growth-promoting effect (figure 1). Until this point has been clarified it can be suspected that also fructose-1,6-diphosphate may play a similar role as that found for glucose-1,6-diphosphate in the metabolism of some organisms.

Regarding the generality of the positive effect of fructose-1,6-diphosphate, nothing definite can be said before more research has been done. Most probably, however, there are many heterotrophic organisms that will respond in the same way as Boletus variegatus and Collybia velutipes. It may also be suggested that under the proper conditions other organic phosphates give similar effects.

Summary

The effect of fructose-1,6-diphosphate on *Boletus variegatus* and two wild type strains L 1 and L 7 of *Collybia velutipes* has been studied.

For both fungi a marked growth-stimulating effect could be recorded at a rather low concentration of the diphosphate. At higher concentrations, the tested compound was growth-inhibiting, at least for Boletus variegatus.

The investigation is only preliminary and offers no explanation of the effect.

References

1. Aschan. K.: Studies on the dediploidisation mycelia of the Basidiomycete *Collybia velutipes.* — Svensk Bot. Tidskr. 46: 366. 1952.

2. Fries, N.: Ueber die Bedeutung von Wuchstoffen für das Wachstum verschiedener Pilze. — Symb. Bot. Ups. 3: 2. 1938.

3. — Ueber die Sporenkeimung bei einigen Gasteromyceten und mykorrhizabildenden Hymenomyceten. — Arch. Mikrobiol. 12: 266. 1941.

4. — Untersuchungen über Sporenkeimung und Mycelentwicklung bodenbewohnender Hymenomyceten. — Symb. Bot. Ups. 6: 4. 1943.

5. Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A. C., and Caputto, R.: The co-enzyme of phosphoglucomutase. — Arch. Biochem. 19: 339. 1948.

6. Leloir, L. F.: in Phosphorus Metabolism I edited by McElroy, W. D. and Glass B. — The Johns Hopkins Press, Baltimore. 1951.

7. Melin, E.: Der Einfluss von Waldstreuextrakten auf das Wachstum von Bodenpilzen mit besonderer Berücksichtigung der Wurzelpilze von Bäumen. — Symb. Bot. Ups. 8: 3. 1946.

8. — Physiology of mycorrhizal relations in plants. — Ann. Rev. Plant. Physiol. 4: 325. 1953.

9. — Growth factor requirements of mycorrhizal fungi of forest trees. — Svensk Bot. Tidskr. 48: 86. 1954.

10. — & Das, V. S. R.: Influence of root metabolites on the growth of tree mycor-rhizal fungi. — Physiol. Plant. 7: 851. 1954.

11. — & Norkrans, B.: Amino acids and the growth of Lactarius deliciosus (L) Fr. — Physiol. Plant. 1: 176. 1948.

12. Norkrans, B.: Studies in growth and cellulolytic enzymes of Tricholoma. — Symb. Bot. Ups. 11: 1. 1950.

13. Schmidt, C. L. A.: The chemistry of amino acids and proteins. — Springfield Ill. 1944.

14. Sutherland, E. W., Colm, M., Posternak, T. & Cori, C. F.: The mechanism of phos-phoglucomutase reaction. — J. Biol. Chem. 180: 1285. 1949.

15. Wiken, T., Keller, H. G., Schelling, C. L. & Stöckli, A.: Ueber die Verwendung von Mycelsuspensionen als Impfmaterial in Wachstumsversuchen mit Pilzen. — Experientia 7: 237. 1951.

Cytophysiological Studies on Micrasterias

IV. Effects of Acids upon the Nuclear Aspect and the Resistance of the Cell

By

Harry Waris

Botanical Institute, University of Helsinki
(Received November 4, 1955)

Introduction

Since acids are largely involved in cell physiology, it would be expected that the treatment of cells with these might affect various phenomena, such as resistance and permeability, photosynthesis, respiration, growth regulation, the cycle of reproduction, and metabolism in general.

In cytological studies with chemical agents, the effects have mostly been studied in fixed material. In the present work, some 40 acids have been tested as to their effects upon the resting nucleus in the living state and upon the resistance of the cell. It appears that, in Micrasterias, weak acids under suitable conditions may cause the resting nucleus to assume a shape similar to prophase. This aspect, called the "grape stage", is characterized by the nucleus, which is nearly spherical at the resting stage, becoming oval and the nucleolar particles liquefied and transformed from irregular grains into smooth globules, which may aggregate into groups suggestive of clusters of grapes (Figure 1; cf. also Waris 1950, Figure 1; Kallio 1951, Figure 1). The fact that a prophase aspect can be artificially induced by weak acids may contribute to the understanding of the chemical changes involved in nuclear and cell division.

The experiments have been carried out with *M. Thomasiana* Archer. *var. notata* (Nordst.) Grönblad. in one case with *M. rotata* (Grev.) Ralfs *var. evoluta* Turner, too.
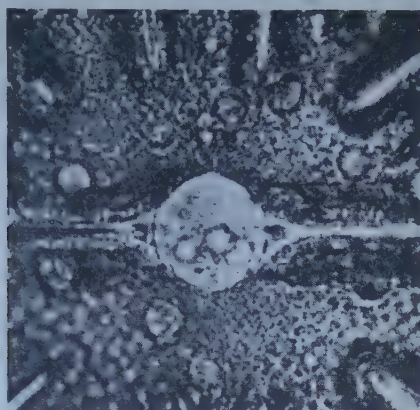
Figure 1. *Micrasterias Thomasiana*. Artificial prophase aspect (grape stage) induced by adipic acid. 1100 ×.

Theoretical Basis of the Experiments

The fundamental difference in penetration power between strong and weak acids has been established by many earlier workers (Brenner 1918, 1920; reviews in Höber 1926, 1946). In general the results have been interpretated in conformity with the lipoid theory, since only the undissociated molecules are in practice able to enter the vacuole. Owing to the different properties of the outer and inner plasma membranes, a distinction has been made beween penetration into the vacuole ("permeability" in a restricted sense) and penetration into the cytoplasmic layer ("intrability" according to Höfler 1932), whereas but little attention has been paied to penetration into the nucleus. This may have been due to methodical difficulties. In Micrasterias, certain responses of the nucleus to acids can easily be perceived, thus making the application of quantitative methods to some degree possible.

Where the effect of hydrogen ions is concerned, the significance of the cell wall as a substrate for adsorption phenomena cannot be ignored. The author has previously shown (Waris 1939) that in the antagonistic effects of metal cations to hydrogen ions the cell wall plays an important part, the protecting effect of the metal cations being mainly restricted to full-grown cells with a mature cell wall, whereas it is hardly noticeable in young cells with a gelatinous, still-growing cell wall. The protecting effect of different cations was found to decrease in the order La > (Ba, Ca, Sr, Mn) > (Rb, Be, Mg) > (K, Cs) > Li > Na. In this connection it is of interest that Höfler and Schindler (1953) have found differences in the adsorptive power of the cell wall even among various species of the same genus, Closterium, and they emphasize, with reference to Czaja (1937), the significance of this in the uptake of nutrients from water.

When we have to do with weak acids, the effects of ions other than the hydrogen ion become important, too. The various alternatives resulting from ionization have been considered by Briggs (1954). A methodical basis is given by the fact that the ionization of weak acids is affected by pH, owing to which their effectiveness can be changed either by varying the pH at constant concentration, or by varying the concentration at constant pH, as has been pointed out by Simon and Beevers (1951, 1952). In attempts to interpret the effects, the concepts "physical" and "chemical toxicity" used by McGowan (1951, 1952 a, b) with reference to earlier workers might be applicable. "Physical toxicity" is assumed to consist in the attainment of a definite concentration of the toxic chemical in a certain "biophase" (Mc-Gowan 1951). Where acids are concerned, chemical reactions may be largely involved, but under given conditions, in particular when the chemical effects of two acids are of the same kind, different results are to be expected if the accumulation of the acids in the biophase differs in magnitude.

In view of the lipoid theory, the assumption seems reasonable that in many cases the biophase concerned is a lipoid one. The degree of accumulation would then depend on the partition of the acid between the "lipophase" and the adjacent "hydrophase". The lipophase may be represented by both the outer and the inner plasma membranes and by some lipoid-containing bodies in the interior of the cell. One must bear in mind that the concepts "lipoid" and "lipoid solubility" are based on indirect evidence, and that water-immiscible substances other than lipoids may also be concerned. The real nature of the lipophase being unknown, the physiological effectiveness of the acids can be compared with partition coefficients calculated for known solvents. Studies on this basis are now much facilitated by the fact that Collander (1947, 1949, 1950, 1951) has summarized most of the available data and completed them by a number of new determinations, some of those considered here not having been published previously.

According to Collander (1947, 1950, 1951), the partition coefficients of homologous substances in two systems of solvents comply with the equation

$$(1) \qquad\qquad \log k_1 = a \cdot \log k_2 + b$$

in which a and b are empirical consants. From this it follows that if we apply the partition coefficients obtained with a definite system of solvents, it should then be possible to find out whether the experimental data are compatible with the partition coefficients in other systems, *i.e.* with partition ratios in general. Thus it is reasonable to compare the present data with the partition coefficients of the acids between ether and water.

The above equation (1) presupposes that some coefficients are known from both systems of solvents to be compared. If the properties of an un-

known solvent such as the plasma lipoid, or any lipophilic phase in the protoplast in general, are to be examined, an indirect method must be applied. This is under certain conditions possible with the weak acids. Two of the most important conditions are the following: a) that a standard response of the cell is related to a certain degree of accumulation of the acid in the lipophilic phase concerned; b) that the surrounding solution can be taken to represent the aqueous phase in the system, or that at least the aqueous phase within the cell is in equilibrium with the surrounding solution. If this is so, then an equation can be derived from which the ratio of the partition coefficients for two acids between the unknown lipophilic phase and the surrounding aqueous solution can be calculated. This ratio can then be compared with that for known solvents.

The equation given by Golumbiç & al. (1949) for monobasic acids can be written in the form (cf. also Hecker 1955, p. 105)

(2 a)
$$k' = \frac{k}{1 + N\log\ (pH - pK)}$$

and that derived by Badgett & al. (1952) for dibasic acids in the form

(2 b)
$$k' = \frac{k}{1 + N\log\ (pH - pK_1) + N\log\ (2pH - pK_1 - pK_2)}$$

where k stands for the partition coefficient of the undissociated molecules between the organic solvent and water, and k′ for that of the total acid, k being independent and k′ dependent on pH and concentration.

Supposing that a certain physiological response is evoked when the accumulation of an acid in an unknown lipophase attains a certain value, then from the data for two acids, a and b, it should be possible to calculate the ratio k_a/k_b of their respective partition coefficients between the unknown lipophase and the adjacent aqueous solution. If $k'_a = k'_b$, it follows from equations (2 a) and (2 b):

(3 a)
$$\frac{k_a}{k_b} = \frac{1 + N\log\ (pH_a - pK_a)}{1 + N\log\ (pH_b - pK_b)}$$

(3 b)
$$\frac{k_a}{k_b} = \frac{1 + N\log\ (pH_a - pK_{1a}) + N\log\ (2pH_a - pK_{1a} - pK_{2a})}{1 + N\log\ (pH_b - pK_{1b}) + N\log\ (2pH_b - pK_{1b} - pK_{2b})}$$

for the mono- and dibasic acids, respectively. Since we do not know whether the ratios k_a/k_b calculated from the experimental data using the above equations actually correspond to the partition ratios of the acids between lipophase and hydrophase, a more neutral symbol Q may be used to denote a value corresponding to the numerator or denominator in the right-hand expressions:

(3 c) $Q = 1 + N \log (pH - pK)$

(3 d) $Q = 1 + N \log (pH - pK_1) + N \log (2pH - pK_1 - pK_2)$

In (3 b), and correspondingly in (3 d), the last term may be negligible at low pH, but significant at high pH.

It appears from equations (2 a) and (2 b) that $Q = k/k'$, $k = k' \cdot Q$, and $k' = k/Q$. Thus the factor Q affects the accumulation of weak acids in all systems of a water-immiscible and aqueous solution, and may be applicable where a quantitative expression for the effectiveness of an acid is required.

On the other hand, the k and k' values for the various acids are only comparable if association or ionization in the lipoid phase and reactions in the aqueous phase can be disregarded. If, for instance, the acid in question appears in the lipophase as double molecules and in the aqueous phase as single molecules, it must be considered that in this case $k = \sqrt{C_o}/C_w$, where C_o denotes the concentration in organic solvent and C_w that in aqueous solution.

If we have some reason to suppose that partition ratios might be responsible for the difference in effectiveness between two acids, there is still another means of checking whether this is probable. Such is the case when the k'/pH curves for the two acids intersect within a physiological range of pH (Figure 2). The intersection depends on the relationship between pK and k, and the point of intersection can be calculated from the following equation:

(4) $$pH_c = pK_a + pK_b + \log \frac{y - 1}{N \log pK_b - y \cdot N \log pK_a}$$

where pH_c denotes the pH for the coinciding k' values, and y the ratio k_a/k_b for the acids a and b. In many cases a simpler equation is applicable:

(5) $$pH = pK + \log \frac{k - k'}{k'}$$

The application of equation (4) may be illustrated by an example (Fig. 2):
Acid a: o-hydroxybenzoic, pK 3.00, k(ether/water) = kE = 229
Acid b: m-hydroxybenzoic, pK 4.12, kE 22

$$pH_c = 3.00 + 4.12 + \log \frac{10.41 - 1}{13182 - 10410} = 4.65$$

At pH 4.65, the coefficients k'E for the acids named attain the value 5.0, and the same holds for p-aminobenzoic acid (pK 4.92, kE 7.73) On the other

hand, the k'E values for the o-, m- and p-aminobenzoic acids do not coincide at any pH value.

For a given pair of acids, the existence of a coinciding points is, of course, also dependent on the organic solvent in question. For instance, if iso-butanol were chosen as solvent, the k' values for the o- and m-hydroxybenzoic acids would not coincide at any pH value, whereas the o-hydroxy- and p-amino-benzoic acids would attain the same k' value at pH 3.86, the k(iso-butanol/water) being for the former 115, for the latter 7.9.

Actually the partition ratios in the living cell cannot be calculated in this way, but the above deductions may be applicable in a reversed way. This means that if the relative effectiveness of two acids, A and B, as compared with one another changes with pH in such a manner that at low values A is more effective than B, at high values, on the contrary, B more effective than A, then the partition ratios seem to be responsible.

Since the solvent properties of weak acids are concerned, attention should be paied to the fact that most of the acids employed are to be regarded as amphiphilic compounds (cf. Winsor 1954). It is, however, not yet possible to evaluate the significance of amphiphily quantitatively.

It is obvious that the application of the above equations implies a considerable simplification of the actual relationships within the cell, as it presupposes that associations of the acid molecules in the unknown lipophase and reactions with the components of the hydrophase do not take place. Actually these conditions may not be fulfilled; on the contrary, reactions between the acids entered and protoplasmic components probably always take place. A further simplification is achieved by using all the ionization constants available as if they were independent of the concentrations of the various components of the solution, which only holds for the thermodynamic constants. However, the last named errors seem to be negligible where solutions of the acids in the same nutrient solution are concerned.

Methods

In most cases, the acid to be tested was added to a nutrient solution to give the concentration desired (mostly 0.002 M), but in some cases the acid or its sodium salt was dissolved in distilled water, the pH being adjusted by addition of 0.1 N HCl or NaOH. Hydrochloric acid was tested as a 0.001 N solution in the presence of 0.001 N CaCl$_2$, which was added to prevent injury to the cell. As nutrient solutions, modifications of the MXS and MS solutions were employed (cf. Waris 1953, p. 540). The modified solutions differed from the original ones, all the heavy metals being supplied as complex salts of ethylene diamine tetraacetic acid (EDTA), the total concentration of which amounted to about 2.8×10^{-5} M. The effect of EDTA was

tested separately at a higher concentration. For the pH determinations, a glass electrode apparatus called the "Autoionometer" (or "Radiometer") was used

For observation, mostly one or a few (3 to 5) cells, sometimes as many as 10 cells or more, with nucleoli presenting the typical resting aspect, *i.e.* coherent masses or irregular grains, were transferred with a glass capillary to watch glasses containing the solution to be tested. A Zeiss 40 $\times$ water immersion objective was then used for close observation. For the latest experiments, a Zeiss-Winkel plankton microscope was available and was found to be very appropriate in combination with the plankton chambers. In many cases it was not possible to follow many cells at the same time, as the changes in individual cells had to be checked at definite intervals. Instead, the results were confirmed by repetition.

The experimental data are given in the following section. As to the physiological responses of the cells, more details will be found in the Discussion.

Constants and Effects of the Acids Tested

The negative logarithms of the ionization constants (pK) are taken from (L) Lange's Handbook of Chemistry, (F & F) Fieser & Fieser (1954), and (D & A) Daniels & Alberty (1955), except that for indole-3-acetic acid, which is taken from Albaum and Kaiser (1937) referring to a 0.0004 M solution. The partition coefficients ether/water for the undissociated molecules (kE values) have been calculated from the data for total partition given by Collander (1949), except for those for the heptoic, caprylic and pelargonic acids, which have been extrapolated from the values for the lower members of the same homologous series (acetic to caproic) according to the equation $y = a + bx + cx^2$, where y denotes log kE and the coefficients a, b and c receive the values —0.29941, 0.545492 and 0.0064236, respectively. The "empirical" values (*i.e.* those calculated for the undissociated molecules on the basis of direct determination of the total partition) and the "calculated" values (obtained from the above equation) are compared below:

	x	kE emp.	kE calc.
Formic	—1	0.42	0.145
Acetic	0	0.52	0.51
Propionic	1	1.80	1.79
n-Butyric	2	6.50	6.56
n-Valeric	3	25	24.8
n-Caproic	4	93	96.6
n-Heptoic	5	—	388
n-Caprylic	6	—	1603
n-Pelargonic	7	—	6820

It will be seen that the "calculated" kE values, except for formic acid, agree reasonably well with the "empirical" ones. With regard to the experimental errors in determining partition coefficients for the higher members of the series, extrapolation may be allowed as giving values which at least express the order of magnitude. In the following, the kE values for caproic, heptoic, caprylic and pelargonic acids have been rounded to 96, 390, 1600 and 6800, respectively.

Abbreviations: k'E partition ratio ether/water for the total amount of acid, kE that for the undissociated molecules. F fusion of the nucleolar particles, G grape stage (! perfect, i incomplete). P signs of irreversible poisoning; (P) slight injury,

such as contraction of the chloroplast; (P?) injury hardly noticeable. D death.
S swelling of the nucleus. L living (n normal appearance, mb morbid). O no visible
changes. h. hour(s), m. minutes after transfer to the acid solution. The pH refers
to the initial value, except for pelargonic acid, where two values, the initial and
the final, are given. Concentration 0.002 M, if not stated otherwise.

The species has been M. Thomasiana v. notata, except for the experiments with
p-hydroxybenzoic acid, where M. rotata v. evoluta, too, has been used.

Carbonic acid

pK_1 6.37, pK_2 10.25 (L), kE $(H_2CO_3 + CO_2)$ 6.8. Carbon dioxide was conducted
through a nutrient solution of pH 6.6 until pH 4.15 was reached. At the latter
pH value (k'E 6.8) a perfect grape stage (G!) was attained within 35—50 m.
The reproductive power was maintained.

Saturated fatty acids $C_nH_{2n}O_2$

Formic, pK 3.75 (L), kE 0.45. At pH 3.8 (k'E 0.22) Gi 45 m.; pH 4.1 (k'E 0.14)
Gi 45 m.; pH 4.5 (k'E 0.07) G! 23—45 m.; pH 6.0 (k'E 0.03) O 4 h. — At the
concentration 0.02 M, pH 2.7 (k'E 0.45) P 24 h.

Acetic, pK 4.76 (L), kE 0.52. At pH 3.9 (k'E 0.46) G! 1—2 h, LGi 24 h., LO 48 h.

Propionic, pK 4.87 (L), kE 1.9 At pH 3.9 (k'E 1.7) G! 2 h., Ln 8 days.

n-Butyric, pK 4.82 (L), kE 6.5. At pH 3.75—4.3 (k'E 6.0—5.0) G! 1/2—2 h.; pH 4.65
(k'E 3.9) G 1/2—2 h.; pH 5.1 (k'E 2.1) Gi 1 1/2 h.; pH 6.4 (k'E 0.17) O 1 1/2 h.

n-Valeric, pK 4.81 (L), kE 26. At pH 3.85 (k'E 23) G! 2—4 h.

n-Caproic, pK 4.85 (F), kE 96. At pH 3.82 (k'E 88) G! 1—4 h., still after 12 h.

n-Heptoic, pK 4.89 (F), kE 390. At pH 3.0 (k'E 385) O 16 m., P 40 m., D 6 h.;
pH 3.5 (k'E 375) O 43 m., P 1 h., D 6 h.; pH 4.1 (k'E 336) F 80 m., P 1—6 h.
(not before 36 m.); pH 4.2 (k'E 324) (F) 1 h., P 3 1/2 h. (not before 1 h.), D 20 h.;
pH 4.3 (k'E 310) S 1 1/2 h., (P) 3 1/2 h. (not before 1 h.), D 20 h.; pH 4.4
(k'E 294) S 1 1/2 h., Ln 3 1/2 h., D 20 h.; pH 4.65 (k'E 248) F 1 1/2 h., Ln 6 1/2 h.,
D 20 h.; pH 5.0 (k'E 170) Gi-G! 1 h., Ln 3 1/2 h.; pH 6.0 (k'E 28) G! 1 h.,
(P) 1—2 h., (P)Gi—G 20 h.

n-Caprylic, pK 4.85 (F & F), kE 1600. At pH 3.85 (k'E 1450) P 5—6 m.; pH 5.0
(kE 664) P 5—9 m., D 1 h.; pH 5.1 (k'E 576) P 30 m., D 3 h. (nuclei coagulated);
pH 5.2 (k'E 494) (P) 1 h., P 3 h.; pH 5.3 (k'E 419) O 1 h., F 3 h., (P) 3 1/2 h.;
pH 5.5 (k'E 292) F(Gi) 3 h., Ln 3 1/2 h., D 22 h.; pH 5.95 (k'E 118) O—Gi 2 h.;
pH 6.6 (k'E 100) G! 35 m., L 46 h.; pH 8.0 (k'E 1.13) F(P) 3 h., Lmb 24 h.;
pH 9.0 (k'E 0.113) G(P) 2 1/2 h. (nucleolar globules few and large, up to 9 μ in
diameter), LF 24 h., LF 42 h.; pH 9.6 (k'E 0.028) G! 3 h. (nucleolar globules
large, 1—5 in number, a single globule 12 μ in diameter), Ln 42 h.

Pelargonic, pK 4.96 (F & F), kE 6800. At pH 5.0—5.2 (k'E 3160—2180) D 5 m.;
pH 6.0—6.2 (k'E 570—370) F(P) 8 m., F(P, single cells D) 6 1/2 h., P 7 h.,
D 13 h.; pH 6.9—7.0 (k'E 97—62) S 1 h., G! 7 1/2 h.; pH 8.0—7.5 (k'E 6.2—19.5)
FSP 9 h.; pH 8.8—7.8 (k'E 1—10) (P) 5 m., FS(P) 8 1/2 h. — N.B. The
solubility of pelargonic acid in water at 20° C. is only 0.026 g/100 g. water
corresponding to a 0.00164 M solution (cf. Seidell and Linke 1952, p. 717).

Substituted fatty acids

Indole-3-acetic acid, pK 4.67 (referring to a determination at 0.0004 M by Albaum
and Kaiser 1937), kE 20. a) IAA added to give 0.002 M, but owing to precipit-

ation the concentration may be lower. At pH 4.0 (k'E 17.6) P 30 m., D 1 $\frac{1}{2}$ h.; pH 5.0 (k'E 7.3) P 45 m., D 1 $\frac{1}{2}$ h.; pH 6.0 (k'E 1.1) O 50 m. (nuclei somewhat opaque) D 24 h.; pH 7.0 (k'E 0.9) SF 1—2 h. (nuclei opalescent), D 24 h.; pH 8.0 (k'E 0.01) SF—G! 35 m. and 1 h. (nuclei opalescent), D 24 h. Destruction commenced with "internal bleeding" (cf. p. 93). — b) 0.0005 M. At pH 4.68 (k'E 9.9) P 2 h. (nuclei opaque, somewhat shrunken, nucleolar grains indistinct), 3 h. (nuclei still more opaque). — c) 0.0002 M. At pH 4.0 (k'E 17.6) O 50 m.; within 9 h. nuclei somewhat opaque, nucleoli indistinct. pH 5.0 (k'E 7.3) G 50 m. — 8 h. in a part of the cells, nuclei somewhat opaque. pH 6.0 (k'E 1.1) Gi 9 h., nuclei opaque, grains indistinct. pH 7.0 (k'E 0.9) SF 45 m., Gi 9 h., nuclei opalescent, oval.

Iodoacetic, pK 3.13 (F & F), kE 7.9. At pH 4.0 (k'E 2.3) FGi 1 h., P 3 $\frac{1}{2}$ h.
Glycolic, pK 3.83 (F & F), kE 0.028. At pH 3.45 (k'E 0.02) G 1 h.
Ethylene diamine tetraacetic acid, pK$_1$ 2.00 (Sequestrene, Alrose Chemical Co.), kE 0.5 (Collander, unpubl.). At pH 4.1 (k'E 0.004) O 45 m., G 24 h.
Lactic, pK 3.87 (F & F), kE 0.10. At pH 4.0 (k'E 0.044) SG! 20 m., D 24 h.

Dicarboxylic acids $C_nH_{2n-2}O_4$

Oxalic, pK$_1$ 1.19, pK$_2$ 4.21 (L), kE 0.12. At pH 4.0 (k'E 0.0001) (P) 5 m., F 2 h.
Malonic, pK$_1$ 2.85 (L), pK$_2$ 5.70 (D & A), kE 0.10. At pH 3.0 (k'E 0.04) P 2 $\frac{1}{2}$ h.; pH 3.9 (k'E 0.008) G 1 h.; pH 4.2 (k'E 0.004) and pH 6.4 (k'E 5×10^{-6}) O 2 $\frac{1}{2}$ h.
Succinic, pK$_1$ 4.19, pK$_2$ 5.57 (L), kE 0.15. At pH 3.5 (k'E 0.12) G 2 h.; pH 4.05 (k'E 0.086) F 2 h.
Glutaric, pK$_1$ 4.33, pK$_2$ 5.57 (F & F), kE 0.28. At pH 3.7 (k'E 0.23) O 4 h., G! 21 h.
Adipic, pK$_1$ 4.43, pK$_2$ 5.52 (F & F), kE 0.55. At pH 3.6 (k'E 0.48) G! 19 h. (Figure 1)
Pimelic, pK$_1$ 4.31 (L), pK$_2$ 5.52 (F & F), kE 1.5. At pH 3.6 (k'E 1.25) G! 17 h.
Suberic, pK$_1$ 4.52, pK$_2$ 5.52 (F & F), kE 4.8. At pH 3.75 (k'E 3.9) G 40 m., P 4 h.
Azelaic, pK$_1$ 4.54, pK$_2$ 5.52 (F & F), kE 16. At pH 3.75 (k'E 14) Gi 75 m.
Sebacic, pK$_1$ 4.55, pK$_2$ 5.52 (F & F), kE 57. At pH 3.7—4.04 (k'E 50—43) P 3 $\frac{1}{2}$ —1 h., D 12 h.; pH 5.0 (k'E 12) D 12 h.; pH 6.0 (k'E 0.50) FLn or D 12 h.

Substituted dicarboxylic acids

Dimethyl malonic, pK 4.15 (L), kE 1.7. At pH 3.85 (k'E 1.1) GiP 5 h., D 16 h.
Diethyl malonic, pK 3.15 (L), kE 6.8. At pH 3.7 (k'E 1.5) Gi 1 $\frac{1}{2}$ h., O 16 h.; pH 3.85 (k'E 1.1) P 5 $\frac{1}{2}$ h.

Aromatic acids

Benzoic, pK 4.17 (F & F), kE 84. a) 0.002 M acid+0.0003 M CaCl$_2$ dissolved in distilled water, pH adjusted with NaOH. At pH 3.5 (k'E 69) O 3 h., Gi (fine grain stage) 4 $\frac{1}{2}$ h.; pH 4.5 (k'E 27), pH 5.1 (k'E 8.8) and pH 6.2 (k'E 0.8) Gi 4 $\frac{1}{2}$ h. (fine grain stage). — b) 0.002 M acid in nutrient solution. At pH 3.5 O 1 h. P (nuclei somewhat shrunken and opaque) 3 h., D 18 h.; pH 4.5 G 30 m., F (nuclei opalescent) 3 h.
o-Hydroxybenzoic, pK 3.00 (F & F), kE 229. At pH 3.0 (k'E 114) D 1 $\frac{1}{2}$ h.; pH 3.5 (k'E 55) P 1 h., D 1 $\frac{1}{2}$—3 h.; pH 4.1 (k'E 17) P 1 h.; pH 4.3 (k'E 11) P 1 $\frac{1}{2}$ h.,

D 19 h. (not before 6 h.); pH 4.5 (k′E 7) D 20 h.; pH 4.65 (k′E 5.0) O or (P) 6 h., L(P) or D 19—24 h.; pH 5.0 (k′E 2.3) F 2 h., (P) 5 h., P or D 19 h.; pH 6.0 (k′E 0.23) Ln 24 h.

m-Hydroxybenzoic, pK 4.12 (F & F), kE 22. At pH 3.5 (k′E 18) Gi 3—4 h., (P)—D 19—24 h.; pH 4.65 (k′E 5.0) O 3 h., L 24 h. and later.; pH 6.0 (k′E 0.3) O 3 h., Ln 24 h. and later.

p-Hydroxybenzoic, pK 4.54 (F & F), kE 26. a) *M. Thomasiana v. notata.* At pH 3.0 (k′E 25) D 12 h.; pH 3.5 (k′E 24) P—D 12 h., D 18 h.; pH 3.8 (k′E 22) Gi—G 3 1/2 h., P—D 19 h.; pH 4.1 (k′E 19) Ln—(P) 12 h., L—D 14 h., D 22 h.; pH 5.0 (k′E 6.7) P—D 11 1/2 h., D 13 h.; pH 6.0 (k′E 0.87) L(P) 19 h.

　　b) *M. rotata v. evoluta.* At pH 3.0 (P) 30 m., P—D 1 h., D 15 h.; pH 3.5 (P) 30 m., P—D 1 h., P—D (most) 18 h.; pH 4.0 (k′E 20) (P) 30 m., F (P) 1 h., P—D 15 h., D 18 h.; pH 5.0 (P?) 30 m., (P) 1—18 h., (P) or D 2 days; pH 6.0 (P) 30 m, (P) 1—18 h, L(P) over 2 days; pH 7.0 (P?) 30 m., Ln 19 h., (P?) over 2 days.

o-Aminobenzoic, pK 5.00 (F & F), kE 28. At pH 3.0 (k′E 28) LnF—(P) 2 h., Gi 4—6 h., L—D 19 h., D 48 h.; pH 3.95 (k′E 26) O 2 h., P 24 h.; pH 4.3 (k′E 23) Gi(P) 1—4 h., L 19 h., D 48 h.; pH 4.65 (k′E 19) Gi 1 h., (P) 19 h. (mucilage very sticky!), D 48 h.; pH 5.0 (k′E 14) Gi 20 m., (P) 2—4 h., LnGi 19—46 h.

m-Aminobenzoic, pK 4.82 (F & F), kE 1.5. At pH 4.1 (k′E 1.26) G! 1 1/2 h., partly receded after 16 h.

p-Aminobenzoic, pK 4.92 (F & F), kE 7.75. At pH 3.5 (k′E 7.5) Gi(P) 12 m., Gi 4 h., D 24 h.; pH 4.1 (k′E 6.7) G! 40 m. and 3 h., D 29.; pH 4.65 (k′E 5.0) Gi 30 m. and 4 h., D 24 h.; pH 6.0 (k′E 0.6) O 30 m., Gi—G(P) 70 m., (P)F (large nucleolar bodies) 4 h., (P)F 24 h.

Gallic, pK 4.40 (F & F), kE 0.51. a) 0.002 *M.* At pH 3.75 (k′E 0.42) O 18 h., but on transfer to normal nutrient solution G! in 24 h; pH 4.55 (k′E 0.21) O 1 1/2 h.; pH 6.0 (k′E 0.013) Gi 25 m., (P) 1 1/2 h. — b) 0.001 *M.* At pH 3.85 (k′E 0.40) L 24 h.

Mandelic, pK 3.37 (L), kE 3. At pH 3.2 (k′E 2) G! 3 h., L 18 h.

Cinnamic (trans), pK 4.43 (L), kE 200 (Collander, unpubl.). At pH 4.0 (k′E 146) P 20 m., D 50 m.

Phenylacetic, pK 4.31 (L), kE 39. a) 0.002 *M.* At pH 3.5 (k′E 34) O 4 1/2 h., P 18 h.; pH 4.0 (k′E 26) O 4 1/2 h., (P) 18 h. — b) 0.001 M. At pH 3.7 (k′E 31) O 3 1/2 h., P 18 h. — c) 0.0001 *M.* At pH 4.5 (k′E 15) O 19 h.

Other acids

Crotonic (trans), pK 4.70 (L), kE 5.4. At pH 4.0 (k′E 4.5) S 7 m., G 1 h.

Hydrazoic, pK 4.59 (L). a) 0.002 *M* as sodium salt. At pH 4.0 O 1 h.; pH 5.0 O 80 m.; — b) 0.15 *M* sodium salt. At pH 6 G 20 h. (1—3 large nucleolar globules)

Hydrochloric. 0.001 *N* HCl+0.001 *N* CaCl$_2$, pH 3.02. A single cell, with a nucleus the size 20×21 μ (diameters in a polar and a transverse direction), was transferred from a nutrient to the acid solution and observed continuously. Nucleus after 4 minutes 23×22 μ, chloroplast contracted; after 5 minutes nucleus 23× 23 μ, nucleolar grains a little more distinct; after 10 minutes grains very fine and rounded; after 45 minutes nucleus a little decreased in size, 21 1/2×21 1/2 μ;

after 24 hours cell still living, nucleus $20 \times 22 \frac{1}{2}$ μ. The maximal increase in volume corresponds to about 40 per cent of the initial volume. The swelling effect was also established in other cases.

Discussion

The physiological responses of Micrasterias to acids can be referred to two categories, reversible and irreversible changes.

Reversible changes often include contraction of the chloroplast, swelling of the nucleus, and liquefaction of the nucleolar particles, which in many cases results in the "grape stage" characteristic of prophase. A typical grape stage further implies that the nucleus becomes oval and transparent-opalescent. The decrease in transparency which often appears on treatment with weak acids, and eventually may result in the nucleus becoming shrunken into a solid body, evidently indicates precipitation and coagulation of the nuclear plasm (including the "nuclear sap"). Careful observation of the successive changes in the nuclear aspect due to weak acids makes it possible to distinguish between the following steps, which correspond to increasing effects: swelling of the nucleus — liquefaction of the nucleolar particles — opalescence — opacity (turbidity) — coagulation of the nuclear plasm (sap).

If an acid which within a certain range of pH is able to induce the grape stage, fails to do so at lower pH values, it seems evident that toxic effects are responsible. Where the acid concerned has a low pK, the toxicity seems primarily to be due to the hydrogen ions introduced by the undissociated molecules into the nucleus and other parts of the cell and freed there by ionization. In many cases, however, the toxicity must depend on specific chemical properties. Thus, in all cases, a chemical toxicity must be involved. The data from the experiments with o-hydroxybenzoic (salicylic) acid indicate that failure to form the grape stage within the range of pH tested is due to the hydrogen ions, as this acid combines a low pK with a high partition ratio organic solvent/water. Similarly, failure to induce the grape stage at the lower end of the pH range with formic, heptoic, caprylic, pelargonic, malonic, sebacic, benzoic acids and others may depend on the hydrogen ion. On the other hand, the increase in toxicity caused by various substitutions must depend on specific chemical properties. As examples may be named the high toxicity of indole-3-acetic and iodoacetic acids, and the difference in toxicity between various benzoic acid derivatives.

To judge from the visible changes, the toxic effects of the weak acids first assert themselves in the nucleus. In many cases, this becomes opaque,

shrunken and coagulated before any visible injury can be discerned in the outer plasma layers or the chloroplast. Death is mostly initiated by an "inner bleeding", which means that the contents of one semicell become pushed towards the other so as to cause the chloroplast of the former to protrude into the isthmus and eventually into the other semicell. The inner bleeding may take place before the chloroplast becomes contracted.

There is a parallelism between my results and those of Brenner (1918, 1920, the former also referred to by Höber 1926, p. 456—458) who found with the red cabbage that weak acids (formic) were able to change the colour before the cells were killed, whereas only at low concentrations could strong acids (hydrochloric, sulphuric) change the colour of living cells; at higher concentrations the colour change only took place after the death of the cells. In this connection the detail of particular interest is that the strong acids tested by Brenner anyway did enter the cells at low concentrations, just as in the present experiments a swelling effect upon the nucleus by hydrochloric acid was established. Consequently, the cell interior may be affected by the strong acids even though there is no severe injury, but it is not possible to decide definitely whether the hydrogen ions (or H_3O^+ ions) as such enter the nucleus, or whether perhaps they suppress the ionization of some weak acids, which pass into the nucleus and accumulate in the lipophilic phase (Winsor, 1954, does not regard the term "hydrophobic" as quite correct). The possibility must be taken into consideration that the nuclear membrane as a water-immiscible phase may play an important part when macromolecular acids, such as the nucleic acids, are concerned.

It seems to be of great significance that any change of pH in the cytoplasm must affect the ionization of the weak acids. According as the relative concentration of the undissociated molecules is changed, the acids may pass from the aqueous into the water-immiscible (lipoid) layers, and *vice versa*. This would seem to constitute an important mechanism of the intracellular phenomena, which must affect the neighbouring cells.

Since an artificial prophase aspect, the grape stage, can be induced by weak acids, the natural phophase, too, may be caused by weak acids. The present results indicate that the liquefying effect of weak acids upon the nucleolar particles is mainly due to the undissociated molecules, the amphiphilic properties of which may be concerned (cf. Winsor 1954). This view is supported by the fact that for the liquefying effect an appropriate relationship between the ionization constant and the partition ratio seems to be required. The existence of a lower limit for the range of pH suitable for the grape stage can be taken as evidence for the view that the liquefying effect is not due to the hydrogen ions, at least not alone, and, on the other hand, the grape stage could not be induced by cyclohexanol, which is a

neutral, ether-soluble substance without any noticeable toxic effects when supplied at the same concentration (0.002 M) as the acids in the present experiments. Thus it seems that the carboxylic group of the acids is essential for the liquefying effect. The swelling effect, on the contrary, seems to be mainly due to the hydrogen ions. Thus the conclusion seems justified that the acids capable of inducing the grape stage act as cosolvents for the nucleolar substance and the nuclear sap, the lipophilic ends attaching to the nucleolar substance, the hydrophilic ends to the nuclear sap. Consequently, the nucleoli in Micrasterias, at the resting stage and prophase, evidently constitute a water-immiscible phase of lipophilic character.

The prophase aspect being thus connected with acidification of the protoplasm, it is to be remembered that in the nuclear cycle a vast complex of chemical changes in nucleus and cytoplasm may be involved, as appears from the reviews by Caspersson (1950), Brachet (1950, 1952), Claude (1950), Ephrussi (1953), Swann (1952), and others. Evidence has been brought forward in support of the view that prophase is connected with an increase in nucleic acids, which seem to take part in protein synthesis. This, in turn, presupposes the presence of amino- and keto-acids, the occurrence of which in plant cells has recently been reviewed by Virtanen (1955) and Miettinen (1955). Moreover, the role of carbon dioxide in photosynthesis, that of various acids in cellular respiration, and the stimulating effect of indoleacetic acid upon nuclear division (cf. Therman 1951) contribute to a confusing multiplicity of interrelations between acids and cellular functions.

As to the grape stage induced by weak acids, the effect has proved to be quite unspecific, the list of the effective acids including carbonic acid (+carbon dioxide) as well as various high-molecular acids. Moreover, Kallio (1951) has seen the grape stage take place on treatment with low temperature. This seems to indicate that enzymes may be involved, as in the case of the sugar-starch equilibrium in potatoes (cf. Bonner 1950). Nevertheless, considering that in the prophase aspect the swelling of the nucleus indicates the action of hydrogen ions, and the liquefaction of the nucleolar particles that of amphiphilic compounds, it appears that both effects can be accounted for on the assumption that weak acids are responsible. This assumption is further supported by the fact that both the liquefying effect upon the nucleoli and the stimulating effect upon mitosis can be exterted by the same weak acid, indoleacetic, although the stimulating effect has not yet been proved with Micrasterias.

In Micrasterias, the biochemical development in the cell between two nuclear divisions seems always to involve acidification, as the grape stage will also result when cell division is inhibited long enough. Thus in cultures in which the nutrient solution is too old, most of the cells eventually present

the grape stage, and similarly this stage appears in cells with a deficient nucleus even it the nutrient solution is faultless (unpubl.). After division, the nucleoli in the daughter cells always present the resting aspect, coherent masses of indistinct particles. Yet even in these young daughter cells, the grape stage can be induced by weak acids. Thus the conclusion seems justified that the reproduction cycle of the cell involves alternating periods of acidification and neutralization, the latter effect being brought about by substances freed from the nucleus during division. In this connection it is interesting that Caspersson (1950) has found the nucleoli to consist of proteins rich in diaminoacids, and that he attributes to the nucleoli a central role in cytoplasmic protein synthesis. Thus the mobilization of the nucleolar substance by the weak acids is of particular interest.

In view of the above considerations, the following hypothesis may be put forward:

A decisive impulse towards nuclear division comes from the cytoplasm in connection with its acidification, which, however, is not the only prerequisite for division. The impulse may be supplied directly or indirectly by some weak acid.

In Micrasterias, cell divisions are concentrated within certain hours of the day, — in large species, as a rule, in the dark period (cf. Kallio 1951, 1953). This may be connected with changes in the carbon dioxide (carbonic acid) concentration in the cell. As to the ability of carbon dioxide (carbonic acid) to liquefy nucleoli, its dissolving power may well depend on the formation of other acids of higher molecular weight. Thus Jacobson (1955) has demonstrated with labeled CO_2 that young excised barley roots were able to fix considerable amounts of it in three hours, the evidence suggesting that the synthesis resulted in the formation of malic acid. Moreover, considering the fact that various amino acids are enzymatically decarboxylated so as to release carbon dioxide (Virtanen 1955), the reverse process also seems possible when the equilibrium is in favour of synhesis. It is evident that various acids may contribute to the acidification of the cell in the dark period.

According to Levan (1940, 1949, 1951) and Levan and Östergren (1943), the spindle apparatus is the substrate affected by the c-mitotic agents, and Levan (1951, p. 237) points out that the nucleus is already affected at prophase, when, according to the current view, the spindle apparatus should not yet be differentiated. Taking into consideration that the stimulating effect of indoleacetic acid also sets in before the spindle is visibly differentiated, namely at the resting stage, the parallelism in the timing of the two effects is striking. The present author, in particular on the basis of cytological data concerning abnormal divisions. believes that the spindle apparatus actu-

ally possesses a structural continuity, the spindle fibres and chromosomes being parts of the same self-reproducing units. This hypothesis would account for the structural changes in the spindle apparatus due to the c-mitotic agents when applied before metaphase. It would also be compatible with the view that the nuclear cycle on the whole is due to chemical changes affecting structural elements.

On the basis of the present data, an attempt can be made at a quantitative estimate of the effectiveness of weak acids. Equation (3) can be applied if the limiting pH values for definite physiological responses can be determined, even if only approximatively. Since at constant concentration the toxicity of a weak acid increases as the pH is lowered, while at a low enough pH the hydrogen ion in itself may be responsible for the toxic effect, the equation can only be applied to acids which exhibit toxicity even at fairly high pH values. The relatively high-molecular acids may fulfil this requirement, provided that there is within the physiologically possible limits a range of pH where they do not exhibit too strong a toxicity. For various reasons, members of the same homologous series seem the most appropriate for such calculations.

In the series of the fatty acids $C_nH_{2n}O_2$, the caprylic and heptoic acids seemed to exert about equal effects at the following pH values:

a) Caprylic pH	5.1	·5.2	5.3	5.5	5.95	6.6
b) Heptoic pH	3.0	4.2	4.3	4.4	5.0	6.0
Q_a/Q_b	2.7	2.7	3.0	4.1	5.9	4.1

The four first-mentioned Q_a/Q_b ratios refer to pH values at which signs of injury have been noted, the two last-mentioned ratios to pH values at which liquefaction of the nucleoli has been established (grape stage at the lower pH less, at the higher more perfect). It may be pointed out that with heptoic acid the grape stage at pH 5.0 was either incomplete or perfect in the various cells, with caprylic acid at pH 5.95 either incomplete or absent, according to which with equal liquefying effects the ratio Q_a/Q_b might be even greater than 5.9. In the system ether/water, the ratio Q_a/Q_b for the acids named is approximately 4 (4.1 has been calculated), and about the same in the system diisopropyl ether/buffer solution (4.3 calculated from Hecker, Table 36). Thus we can conclude that the difference in effectiveness between caprylic and heptoic acids might possibly be accounted for by partition ratios lipophase/hydrophase about 3 to 6 times as high for caprylic as for heptoic acid, and that the corresponding ratios in the system ether/water fall within these limits.

A similar calculation of the Q_a/Q_b ratios for the pelargonic and caprylic acids give a value of about 2 (1.9—2.2), but the water-solubility of the pelar-
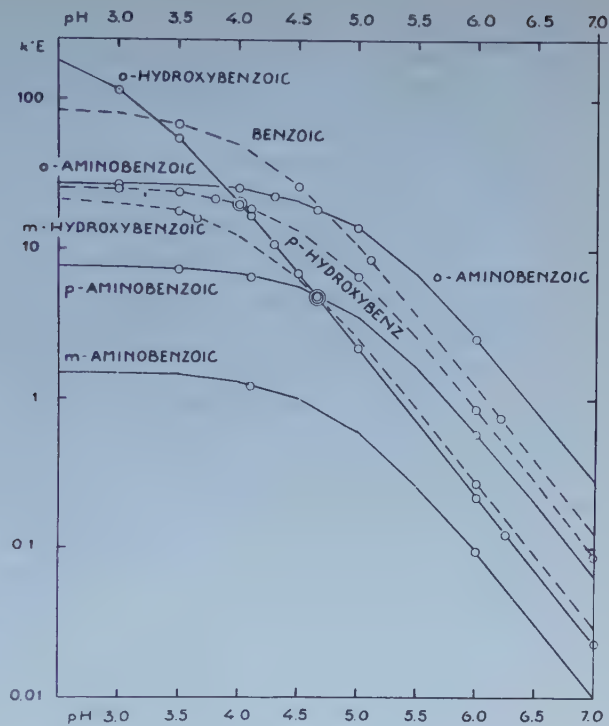
Figure 2. *k'E/pH curves of benzoic acid and its derivatives. Circles refer to the pH values tested.*

gonic acid being very low (0.0016 M at 20° C), the estimation of the corresponding pH values seems still more uncertain.

If non-homologous acids are compared, no parallelism will be found between toxicity and the partition ratios ether/water. Instead, the degree of toxicity can be expressed by the Q values limiting for certain physiological responces, low Q values indicating low, high Q values high toxicity. Here the limiting Q value refers to the lowest value allowing the response to take place.

For instance, in the homologous series of the saturated fatty acids $C_nH_{2n}O_2$ the members from acetic to caproic may induce a perfect grape stage below Q 1.1, whereas heptoic requires at least Q 2, caprylic Q 14, and pelargonic still more. If we compare the dicarboxylic acids $C_nH_{2n-2}O_4$ with the fatty acids named, it appears that a part of the cells may remain alive for 12—14 hours when the pH is about 6 with sebacic acid, between 5.5 and 6 with caprylic acid, and between 4.65 and 6 with heptoic acid. The corresponding Q values are 114, 5.5—15, and 1.6—14, respectively. This implies that at the same concentration (0.002 M) sebacic acid is much more toxic than the caprylic and heptoic acids. It seems obvious that the higher toxicity of the dicarboxylic acids as compared with the monocarboxylic acids depends on

7

the lower pK values of the former. The coagulation of the nuclear plasm indicates that the action of the hydrogen ion is responsible.

Thus the Q factor offers an appropriate measure for the effectiveness of acids at constant concentration.

Another means of comparing the effectiveness of the acids with partition coefficients is exemplified by the k'E/pH curves of the benzoic acid derivatives (Fig. 2), the points of intersection of which can be determined by equation (4). From the intersecting curves it may be gathered that if the change of pH over a certain range involves a reversion of the relative effectiveness of two acids, partition coefficients may be responsible for the change. The experiments with M. Thomasiana v. notata indicated, in fact, that at low pH (3 to 4) the o-hydroxybenzoic acid is more toxic than the p-hydroxy acid, whereas at high pH (6) the latter seemed to be a little more toxic than the former. Similarly, at low pH the o-hydroxy acid was definitely more toxic, at high pH a little less toxic than the p-amino acic. It may be noted that at high pH a great difference in toxicity is not even to be expected. On the other hand, at all pH values tested (3 to 7) the o-hydroxy acid was more toxic to M. rotata v. evoluta than the p-hydroxy acid. Thus the two species showed a different sensitivity to the acids concerned.

Besides those already mentioned, the following specific effects may be of interest. The amino derivatives of benzoic acid seem to have a greater tendency to liquefy nucleoli than the hydroxy derivatives. Among the effects of the o-aminobenzoic acid, the exceptional stickiness of the mucilage was striking. With gallic acid, the delay in the appearance of the grape stage until after transfer to normal nutrient solution indicates that in this case oxydation-reduction phenomena are involved.

Irrespective of the lack of parallelism between effectiveness and partition ratios in many cases, the relationship between effectiveness and pH with on and the same acid clearly shows that the final effect depends on the ionization equilibrium in the surrounding solution, the concentration of the undissociated molecules being decisive for the effect below the neutral point at least. There is, however, some evidence in favour of the view that at alkaline pH values the anions of the high-molecular fatty acids contribute to the liquefaction of nucleolar particles, as with caprylic acid at pH 8 and 9.6 the fusion of these often resulted in exceptionally large, though not always regular, bodies.

In general, a high partition ratio ether/water implies toxicity, the acids with the highest kE values, such as o-hydrobenzoic, cinnamic, caprylic and heptoic acids, being among the most toxic experimented with. A low pK when combined with a high kE evidently contributes to the toxicity (o-hydrobenzoic, sebacic), but may be tolerated when the kE is low enough

(mandelic acid). Thus the partition between the water-immiscible and aqueous phases in the cell must be of prime importance for the effectiveness of the weak acids in general, although the part played by the partition ratios in the difference in effectiveness between many acids might be masked by specific chemical effects.

Summary

Some 40 weak acids have been tested as to their effect upon the nucleus at the resting stage and upon the resistance of the cells. The method of varied pH at constant concentration has been applied.

All weak acids, provided they are soluble enough and toxic effects do not interfere, seem to be capable of liquefying the nucleoli so as to cause the "grape stage" characteristic of prophase. A distinction can be made between the swelling of the nucleus and the liquefaction of the nucleoli, the former being mainly caused by the hydrogen ions, the latter by the undissociated molecules and, at high pH values, possibly also by the anions.

The liquefying effect of the acid molecules seems to be due to the fact that, as amphiphilic compounds, they act as cosolvent for the nucleolar substance and nuclear sap, the lipophilic ends of the molecules attaching to the former, the hydrophilic ends to the latter substance. Consequently the nucleoli constitute a lipophilic phase in the cell.

Within the homologous series of the saturated fatty acids $C_nH_{2n}O_2$ and the dicarboxylic acids $C_nH_{2n-2}O_4$, the toxicity increases with the partition ratio ether/water. In general, a high partition ratio ether/water implies toxicity. A low pK when combined with a high partition ratio contributes to the toxicity, but may, perhaps, be tolerated when combined with a low partition ratio.

When non-homologous acids are compared as regards their effectiveness, the part played by the partition ratios is largely masked by that of specific chemical effects.

Some equations have been derived in order to elucidate the significance of partition ratios.

The hypothesis has been put forward that the onset of nuclear division may receive some decisive impulse from the cytoplasm, the acidification of which may cause some weak acids to pass into the nucleus.

A relationship is suggested between the concentration of cell divisions within definite hours of the day and the carbon dioxide concentration in the cell.

References

Albaum, H. G. & Kaiser, S.: The titration curves of 3-indole acetic, 3-indole propionic, and 3-indole butyric acids. — Amer. J. Bot. 24: 420. 1937.

Badgett, C. O., Eisner, A. & Walens, H. A.: Distributions of pyridine alkaloids in the system buffer-t-amyl alcohol. — J. Amer. Chem. Soc. 74: 4096. 1952.

Bonner, J.: Plant Biochemistry. — New York 1950.

Brachet, J.: The localization and the role of ribonucleic acid in the cell. — Ann. New York Acad. Sci. 50: 862. 1950.

— The role of the nucleus and the cytoplasm in synthesis and morphogenesis. — Symp. Soc. Exp. Biol. 6: 173. 1952.

Brenner, W.: Studien über die Empfindlichkeit und Permeabilität pflanzlicher Protoplasten für Säuren und Basen. — Öfversigt af Finska Vetenskaps-Societetens Förhandlingar 60: 1. 1918.

— Über die Wirkung von Neutralsalzen auf die Säureresistenz, Permeabilität und Lebensdauer der Protoplasten. — Ber. deutsch. bot. Ges. 38: 277. 1920.

Briggs, G. E.: The relation between concentration of hydrogen ions and the effect of weak acids or bases on metabolic activity. — J. Exp. Bot. 5: 263. 1954.

Caspersson, T.: Cell Growth and Cell Formation. — New York 1950.

Claude, A.: Studies on cell morphology and functions: methods and results. — Ann. New York Acad. Sci. 50: 854. 1950.

Collander, R.: On lipoid solubility. — Acta Physiol. Scand. 13: 363. 1947.

— Die Verteilung organischer Verbindungen zwischen Äther und Wasser. — Acta Chem. Scand. 3: 717. 1949.

— The distribution of organic compounds between iso-butanol and water. — Ibidem 4: 1085. 1950.

— The partition of organic compounds between higher alcohols and water. — Ibidem 5: 774. 1951.

Czaja, A. Th.: Untersuchungen über den Membraneffekt des Absorptionsgewebes und über die Farbsoffaufnahme in die lebende Zelle. — Planta 26: 90. 1937.

Daniels, F. & Alberty, R. A.: Physical Chemistry. — New York and London 1955.

Ephrussi, B.: Nucleo-Cytoplasmic Relations in Micro-Organisms. — Oxford 1953.

Fieser, L. F. & Fieser, M.: Lehrbuch der organischen Chemie. — Weinheim 1954.

Golumbic, C., Orchin, M. & Weller, S.: Partition studies on phenols. I. Relations between partition coefficient and ionization constant. — J. Amer. Chem. Soc. 71: 2624. 1949.

Hecker, E.: Verteilungsverfahren im Laboratorium. — Weinheim 1955.

Höber, R.: Physikalische Chemie der Zelle und der Gewebe. — Leipzig 1926.

— Physical Chemistry of Cells and Tissues. — Philadelphia 1946.

Höfler, K.: Zur Tonoplastenfrage. — Protoplasma 15: 462. 1932.

— & Schindler, H.: Vitalfärbbarkeit verschiedener Closterien. — Protoplasma 42: 296. 1953.

Jacobson, L.: Carbon dioxide fixation and ion absorption in barley roots. — Plant Physiology 30: 264. 1955.

Kallio, P.: The significance of nuclear quantity in the genus Micrasterias. — Ann. Bot. Soc. "Vanamo" 24. No. 2. 1951.

— The effect of continued illumination on the desmids. — Arch. Soc. "Vanamo" 8: 58. 1953.

Lange, N. A.: Handbook of Chemistry. — Sandusky 1952.

Levan, A.: The effect of acenaphthene and colchicine on mitosis of Allium and Colchicum. — Hereditas 26: 262. 1940.

— The influence on chromosomes and mitosis of chemicals, as studied by the Allium test. — Proc. Eighth Intern. Congress of Genetics (Hereditas Suppl. p. 325). 1949.

— Chemically induced chromosome reactions in Allium cepa and Vicia faba. — Cold Spring Harbor Symp. on Quantitative Biology 16: 233. 1951.

— & Östergren, G.: The mechanism of c-mitotic action. — Hereditas 29 :381. 1943.

McGowan, J. C.: The physical toxicity of chemicals. I. Vapours. — J. Applied Chem. 1: S 120. 1951.

— The physical toxicity of chemicals. II. Factors affecting physical toxicity in aqueous solutions. — Ibidem 2: 323. 1952 a.

— The physical toxicity of chemicals. III. A systematic treatment of physical toxicity in aqueous solutions. — Ibidem 2: 651. 1952 b.

Miettinen, J.: Free amino acids in the pea plant (Pisum sativum). — Biochemistry of Nitrogen. Ann. Acad. Sci. Fenn. A II. 60: 520. 1955.

Seidell, A. & Linke, W.: Solubilities of Inorganic and Organic Compounds. Suppl. to the third Edition. — New York 1952.

Simon, E. W. & Beevers, H.: The quantitative relationship between pH and the activity of weak acids and bases in biological experiments. — Science 114: 124. 1951.

— — The effect of pH on the biological activities of weak acids and bases I. The most usual relationship between pH and activity. — The New Phytologist 51: 163. 1952 a.

— — The effect of pH on the biological activities of weak acids and bases. II. Other relationships between pH and activity. — Ibidem 51: 191. 1952 b.

Swann, M. M.: The nucleus in fertilization, mitosis and cell-division. — Symp. Soc. Exp. Biol. 6: 89. 1952.

Therman, E.: The effect of indole-3-acetic acid on resting plant nuclei I. Allium cepa. — Ann. Acad. Sci. Fenn. A IV. 16: 1. 1951.

Virtanen, A. I.: Neue Amino- und Ketosäuren in grünen Planzen und die Biosynthese der Aminosäuren. — Angew. Chemie 67: 381. 1955.

Waris, H.: Über den Antagonismus von Wasserstoffionen und Metallkationen bei Micrasterias. — Acta bot. fenn. 24: 1. 1939.

— Cytophysiological studies on Micrasterias I. Nuclear and cell division. — Physiol. Plant. 3: 1. 1950.

— The significance for algae of chelating substances in the nutrient solutions. — Ibidem 6: 538. 1953.

Winsor, P. A.: Solvent Properties of Amphiphilic Compounds. — London 1954.

The Uptake of Auxin by Plant Tissue [1]

By

Mary Perrine Johnson and James Bonner

Kerckhoff Laboratories of Biology, California Institute of Technology,
Pasadena, Calif.
(Received October 29, 1955)

It is known that the application of an auxin such as indoleacetic acid (IAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) to plant tissue evokes a growth response. It is not clear however to what extent the penetration and absorption of the auxin into the tissue regulates the growth response. Data on the uptake of an auxin, 2,4-D, by sections of the Avena coleoptile, will be presented in the present report.

When auxin is supplied to the coleoptile, it enters the tissue and may be subsequently in part recovered by solvent extraction (2). It is of obvious interest to know how the concentration inside the tissue is related to the concentration of the external solution. Is the auxin response of coleoptile tissue related to internal auxin concentration or to the concentration of the external solution? This might be determined by finding out, for example, whether the auxin concentration inside the tissue exhibits the same sort of concentration dependence on external concentration as the final growth rate itself (Foster *et al.* 5). It will be shown below that auxin given in the external solution enters the Avena coleoptile section by two separable processes. The first process is a rapid one consummated in 20—30 minutes, and itself consists of two separable components. The second is a continuous active absorption, which resembles the active accumulation of ions by plant tissues. Neither process can as yet be directly related to the auxin-induced growth response.

Materials and Methods

Avena seedlings (var. Siegeshafer) were grown in stainless steel trays in a medium of vermiculite as described by McRae and Bonner (6) They were grown for 92—96 hours under low intensity red light at 25° C and 90 per cent humidity. Coleoptiles 2.50—3.25 cm in length were selected for use, and a 5 mm section was cut 2—3 cm from the tip of each coleoptile. It was found that the leaves do not take up 2,4-D appreciably, and they were therefore not removed from the sections. The presence of the leaf prevents the 2,4-D solution from being trapped in the central hole of the sections. Pooled and randomized sections were distributed in lots of 40 or 60 per treatment.

The basal nutrient solution contained 0.0025 M potassium maleate buffer (pH 4.5) and distilled water unless otherwise specified. For these short-time experiments, sucrose was not provided in the basal medium. The C^{14} carboxyl labeled 2,4-D (10 m curies/m mole, synthesized by Tracer Lab. Inc.) was made up in 100.0 mg/l stock solution. This stock solution was neutralized with potassium hydroxide (unless otherwise noted) and added to the basal medium in appropriate concentration. Five ml of solution per 60×15 mm Petri dish per 60 sections was established as a sufficiently high ratio of solution to sections to maintain an essentially constant external 2,4-D concentration over the experimental period.

During incubation, the Avena coleoptile sections floated on the surface of the medium and were maintained in darkness with occasional orange light. At the end of the experimental period, the sections were poured onto a plastic screen and washed for approximately five seconds with distilled water before removal in lots of 20 to the counting planchets. The 60 sections of a single treatment were washed and placed on planchets within one minute. Once the sections were arranged on the counting plates, they were dried under an infra-red heat lamp.

The amount of radioactivity contained in the coleoptile tissue after incubation was determined by counting either with a Geiger-Müller end window tube or with a Geiger tube equipped with a micromil window and operated in an atmosphere of Q gas. The gas flow tube has approximately 6 times the sensitivity of the thin window tube. Counts per minute minus background (CPM) were computed for each planchet. Experimental values are based on duplicate count of each of two to four replicate samples of 20 sections.

The sensitivity of the counting tubes is uniform over the area of the planchet, 2 8 cm². It is unnecessary, therefore to arrange individual sections into a given pattern on the planchet. Provided that the sections are in a monosectional layer, the radioactivity found is linear with the number of sections counted, as shown in table 1. When the planchet contains several layers of sections, CPM are decreased due to the increased self-absorption of the tissue. If the tissue is ground and counted, the activity is identical with that obtained from whole sections (table 1). The activity at infinite thickness calculated from the data for monosectional layers is also within 15 per cent of the values calculated from counts of layers of ground tissue. The radioactivity data presented below, other than that of tables 1 and 3, are not corrected for self absorption unless specifically noted.

In experiments of the duration considered here, the 2,4-D taken up by the coleoptile is not metabolized to any considerable degree. This is shown by experiments in which sections were incubated for three hours in labeled 2,4-D, 2 mg/l. At the end of three hours the sections were lyophilized, ground, and the dry material

Table 1. *Counting characteristics of Avena coleoptile sections which have absorbed C^{14} labeled 2,4-D. All sections counted on planchets of area 2.8 cm^2.*

No. of sections	Wt. in mg	Area covered cm^2	mg/cm^2	CPM	CPM (infinite thickness) /sample/ 2.8 cm^2
10[a]	2.1	0.375	5.6	132	1,760
20[a]	4.2	0.75	5.6	281	1,880
50[a]	10.5	1.875	5.6	670	1.790
200[b]	40.6	2.80	14.5	1,229	1,540
145[c]	30.4	2.80	10.8	1,104	1,520

a Monosectional layer. b Multisectional layer. c Sections ground to a powder.

Table 2. *Sources of variability in determination of uptake of labeled 2,4-D by Avena coleoptile sections. Sections incubated 120 min in 2,4-D, 0.5 mg/l.*

Lot (40 sections)	Sample (20 sections)	CPM per 20 sections			
		Count 1	Count 2	Av. of replicates	
				Counts of 20	Samples of 20
1	1	90	93	91.5	—
	2	98	98	98	95
2	1	96	102	99	—
	2	98	100	99	99
3	1	106	106	106	—
	2	94	101	97.5	102
4	1	101	103	102	—
	2	103	101	102	102
5	1	93	91	92	—
	2	94	90	92	92
6	1	101	100	101.5	—
	2	89	99	94	98
7	1	87	91	89	—
	2	98	101	99.5	94
8	1	98	101	99.5	—
	2	95	95	95	97
9	1	101	100	100.5	—
	2	102	103	102.5	102
10	1	96	103	99.5	—
	2	112	103	107.5	103
				Av. of lots	98.5

exhaustively extracted with 80 per cent aqueous acetone. Of the radioactivity present in the ground tissue, 99.8 per cent was removed in this way. The whole acetone extract was concentrated, applied to paper, and the chromatogram developed in isopropanol; ammonia; water (10 : 1 : 1). After drying, a radioautograph was made from the chromatogram on non-screen x-ray film. All detectable radioactivity was contained in a single spot (R_F 0.70—0.72) corresponding to known 2,4-D.

The sources of variability in the present type of experiment are considered in the data of table 2. For this experiment 10 replicate lots of 40 sections each were allowed to take up labeled 2,4-D (0.5 mg/l) for 120 minutes. Each lot was then washed, divided into 2 samples of 20 sections each, and each sample counted twice. The data of table 2 show that replicability of counts on a single sample and replicability of samples within a single lot is greater than replicability between lots. It is evident that little is to be gained by further counting or by multiplication of samples. The standard deviation of activity of all lots is 3.9 per cent of the mean, and approximately 95 per cent of all individual lot counts may be expected to lie within the range ± 7.8 per cent of the mean. The data of this paper consist in general however of lines based on several points, each point having the above precision of measurement. In addition, each experiment has been repeated 2 to many times.

Experimental Results

Progress Curves for 2,4-D Uptake: Figure 1-A gives data on the uptake by coleoptile sections of 2,4-D supplied at an external concentration somewhat less than that optimal for growth (0.5 mg/l, 2.3×10^{-6} M). A brief initial period of rapid uptake is followed by a period of slower absorption which continues for six hours or more without decrease in rate. The uptake of 2,4-D by coleoptile sections is evidently composed of two separate processes, an initial rapid uptake which is consummated in 20—30 minutes and a continuing absorption which is constant with time over longer periods. Since the continuing absorption is linear with time, it is possible to use the ordinate intercept of the progress curve as a measure of the total 2,4-D taken up by the initial rapid process. Progress curves for the uptake of 2,4-D by the two individual processes are plotted in figures 1-B and 1-C. Qualitatively this uptake of 2,4-D by coleoptile tissue resembles the uptake of inorganic ions by roots or by storage tissue. In these cases also the absorption may be separated into an initial rapid process and a continuing absorption (9).

Progress curves for uptake of 2,4-D from a wide range of 2,4-D concentrations are shown in figure 2-A. From these data the concentration dependencies of the two kinds of 2,4-D uptake may be separately evaluted. They are given in figure 2-B. Total initial uptake (as measured by the ordinate intercepts of the progress curves) is essentially linear with 2,4-D concentration over the wide range considered here. The continuing uptake of 2,4-D (as measured by the slopes of the straight lines of figure 2-A) also approaches linearity at the higher concentrations (Figure 2-B).

That the continuing uptake is an actual accumulation of 2,4-D is shown by the data of table 3. Over an incubation period of 6 hours, 2,4-D is accumulated in the tissue to a concentration higher than the external concentration.
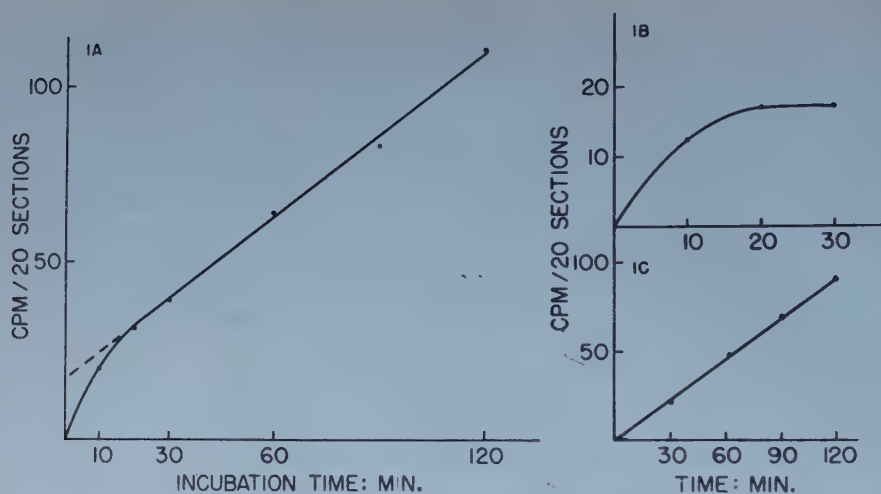
Figure 1. *Progress curves for uptake of C^{14} carboxyl labeled 2,4-D by Avena coleoptile sections.*

Figure 1-A. C^{14} *carboxyl labeled 2,4-D content of sections incubated for various times in 2,4-D, 0.5 mg. l. The dotted line is that extrapolated from the linear continuing portion of the curve. The ordinant intercept represents the total 2,4-D taken up by the initial rapid process.*

Figure 1-B. *Progress curve for uptake of C^{14} carboxyl labeled 2,4-D by the initial rapid process. Data from Figure 1-A.*

Figure 1-C. *Progress curve for the uptake of C^{14} carboxyl labeled 2,4-D by the continuing absorption. Data from Figure 1-A.*
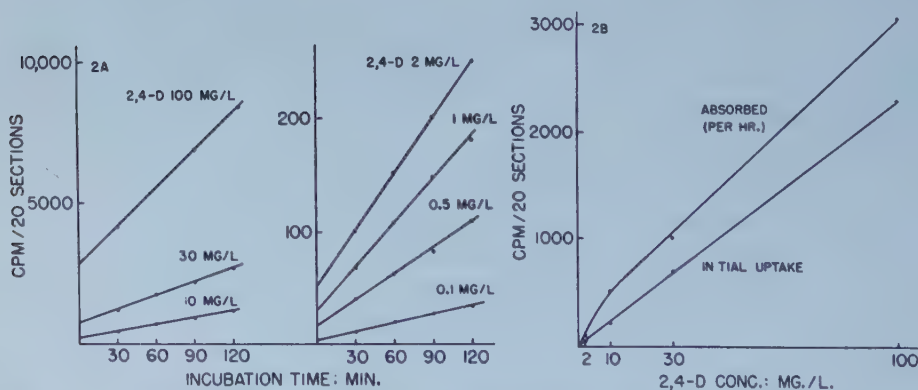
Figure 2. *Progress curves for uptake of C^{14} carboxyl labeled 2 4-D by Avena coleoptile sections.*

Figure 2-A. Left. *Data for high concentrations of 2,4-D, 10 to 100 mg/l.*
Right. *Data for low concentrations of 2,4-D, 0.1 to 2 mg./l.*

Figure 2-B. *Uptake of 2,4-D by Avena coleoptile sections as a function of 2,4-D concentration. Upper curve, continuing absorption. Lower curve, initial uptake.*

Table 3. *Accumulation of 2,4-D by Avena coleoptile sections during incubation for 6 hrs. at 25° C. All counts calculated to infinite thinness.*

External 2,4-D conc.		2,4-D conc. in sections CPM/100 mg fresh wt
mg/l	CPM/0.1 ml sol'n.	
0.1	43	293
0.5	216	618
1.0	433	1,240
2.0	866	2,470

Table 4 *Amount of 2,4 D removed by 60 minutes leaching in buffer from sections previously incubated for 30 minutes in varied concentrations of labeled 2,4-D. The counts of the sections are corrected to the same sample thickness as that of the solution. (gas flow counting)*

Solution used for incubation		Counts 2,4-D removed by leaching		Diffusible volume C_I/C_0
2,4-D conc. mg/l	CPM/0.1 ml (C_0)	CPM 20 sect.	CPM/100 mg Corr. to inf. thinness (C_I)	
0.5	1,300	98	280	0.22
1.0	2,600	175	503	0.19
2.0	5,200	400	1,145	0.22
10.0	26,000	2,000	5,750	0.22

At least a portion of the 2,4-D which enters the tissue during the initial rapid process appears to do so by diffusion. This is shown by experiments in which sections were allowed to take up labeled 2,4-D for 30 minutes and were then transferred to buffer lacking 2,4-D. Labeled 2,4-D is lost by the sections to the buffer. This process is largely completed within 30 minutes and comes to a definite end point (figure 3). Table 4 gives data on the amount of labeled 2,4-D which is removed during 60 minutes in buffer after a 30 minute pretreatment in 2,4-D of varying concentration. The amount of 2,4-D which leaches out is approximately proportional to the concentration of 2,4-D in which the sections have been previously incubated. Let us suppose that the 2,4 D which is free to diffuse out of the section is 2,4-D which originally diffused into the tissue. Since the fresh weight of the sections is known (70 mg./20 sections), we can calculate what fraction of this weight is in apparent diffusion equilibrium with the external solution. The data of table 4 indicate that this accessible or apparent free space of the tissue is 19—22 per cent.

That the initial uptake of 2,4-D consist not only of diffusion but also of a second process is indicated by the data of figure 3-A. Tissue was incubated in labeled 2,4-D, 0.5 mg/l, for thirty minutes and then transferred after washing to a solution containing either buffer and unlabeled 2,4-D or buffer
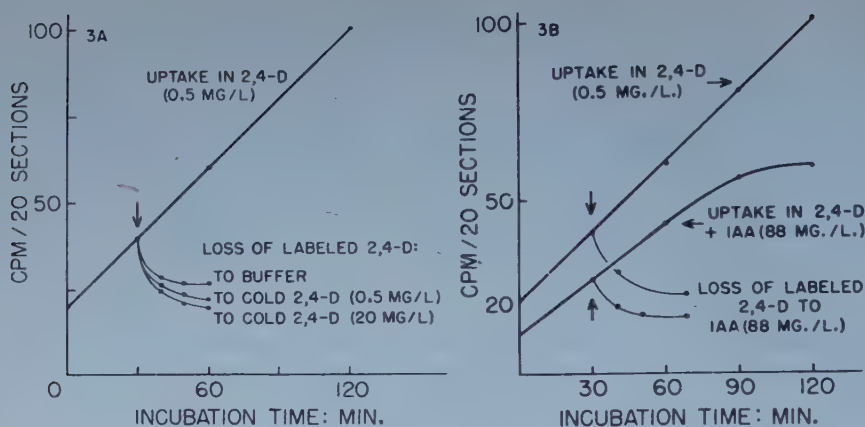
Figure 3. *Loss of C¹⁴ carboxyl labeled 2,4-D from Avena coleoptile sections to solutions of various compositions.* Sections incubated in C-14 carboxyl labeled 2,4-D 0.5 mg./l. After 30 minutes incubation (indicated by the arrow) a portion of the sections was moved to *unlabeled solution.*

Figure 3-A. *Loss of labeled 2,4-D to buffer in presence or absence of unlabeled 2,4-D.*

Figure 3-B. *Loss of labeled 2,4-D to IAA solution* (upper curve) *or uptake and loss of labeled 2,4-D in the presence of a high concentration of IAA.*

alone. Loss of 2,4-D from the tissue approaches completion after 30 minutes as noted above. The loss of tissue 2,4-D to the external solution is less to solution containing buffer alone than it is to solution containing buffer and unlabeled 2,4-D. The quantity of labeled 2,4-D lost by the tissue in 30 minutes to a solution containing an excess (20 mg/l) of unlabeled 2,4-D just equals the total 2,4-D taken up by the initial rapid process. The amount of 2,4-D lost to the buffer alone is less than the total taken up by the initial process. The 2,4-D lost to buffer is equivalent to that which would be contained in 0.18 of the tissue wet weight if the tissue were in diffusion equilibrium with the external solution. This is in approximate agreement with the calculation of table 4.

That initial uptake of 2,4-D by coleoptile sections consists of two separable processes is indicated also by the experiment of figure 3-B, summarized in table 5. For this experiment, sections were incubated in 2,4-D, 0.5 mg/l (2.3 $\times 10^{-6}$ M), in the presence or absence of an excess of IAA, 88 mg/l, $(5 \times 10^{-4}$ $M)$. After 30 minutes sections were washed and placed in solution containing IAA $(5 \times 10^{-4}$ $M)$. The 2,4-D taken up in the initial process by sections incubated in the absence of IAA was essentially completely lost again to the IAA solution. Sections incubated in 2,4-D and IAA took up less 2,4-D by the initial process than did those incubated in 2,4-D alone. The
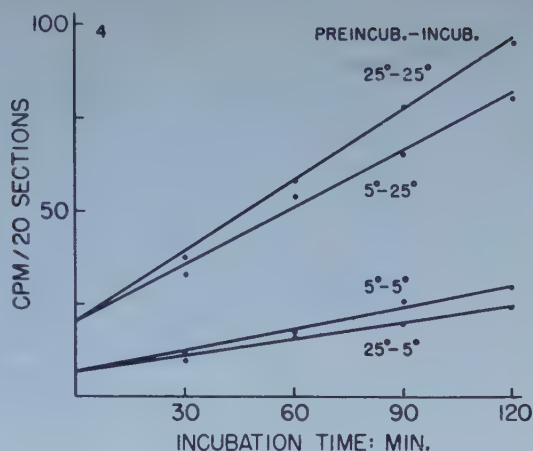
Figure 4. *Progress curves for uptake of C^{14} carboxyl labeled 2,4-D by Avena coleoptile sections at $25°$ and $5°$. In each case sections were pretreated at $25°$ or $5°$ before transfer to the incubation mixtures.*

Table 5. *Effect of IAA in high concentration on initial uptake and on outward diffusion of 2,4-D. Coleoptile sections incubated in 2,4-D 0.5 mg/l. IAA, 88 mg/l.*

Incubation sol'n.	CPM/20 sections	
	Initial process 2,4-D taken up	2,4-D lost to solution in presence of IAA, 88 mg/l
2,4-D alone	23	21
2,4-D + IAA	11	12

amount taken up in the presence of IAA equals that shown in table 4 to be contained in the section in a diffusible state. The presence of an excess of IAA in the solution does not therefore influence the amount of 2,4-D passing into the tissue by diffusion. The binding of 2,4-D in the exchangeable form appears however to be suppressed by IAA in high concentration. It may be calculated from the data of table 5 that, in the presence of IAA, 0.16 of the tissue is available for the entry of 2,4-D by diffusion. This is in approximate agreement with the values calculated from table 4 and figure 3-A.

Effects of Temperature on 2,4-D Uptake: Progress curves for 2,4-D uptake at $25°$ C and at $5°$ C are shown in figure 4. Both the amount of initial uptake and rate of continued uptake are reduced at the lower temperature. Pretreatment of coleoptile sections in buffer at $5°$ C for one to four hours does not greatly change rate of subsequent 2,4-D uptake at $25°$ C or $5°$ C from that which occurs with non-cold treated sections. Both aspects of the uptake appear to be primarily affected by the temperature at which the actual uptake of 2,4-D takes place and are not permanently affected by pre-treatment at low temperature.
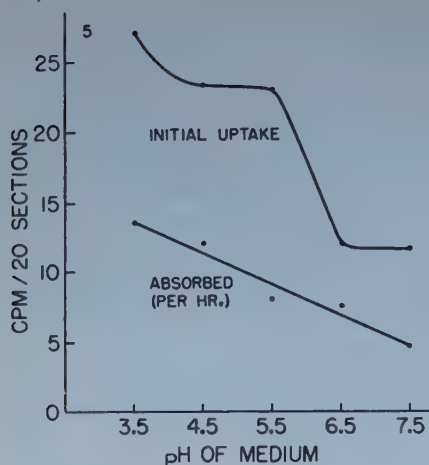
Figure 5. *Uptake of C^{14} carboxyl labeled 2,4-D by Avena coleoptile sections as a function of external pH. 0.0025 M K-maleate buffer. Upper curve, amount of 2,4-D taken up by the initial process as a function of pH. Lower curve, rate of continuing absorption as a function of external pH.*

pH Dependence of 2,4-D Uptake: The uptake of 2,4-D by Avena coleoptile tissue is related to the pH of the external solution in the manner shown in figure 5. For these experiments 0.0025 *M* K-maleate buffer was made up to the desired pH. Both the initial uptake and the rate of the continued uptake of 2,4-D are decreased as the pH of the external medium is increased. This decrease amounts to approximately 2.5 fold over the range pH 4.5—7.5 in both instances. It is possible that the relative amounts of diffusible and exchangeable 2,4-D taken up by the tissue may change as the pH is increased. This is suggested by the fact that the total initial uptake at pH 7.5, if all due to diffusion, would yield a diffusible volume of 0.15 the weight of the tissue. Whether the exchangeable binding of 2,4-D is in fact suppressed at high pH has not been investigated further.

Effect of Metabolic Inhibitors on Uptake of 2,4-D: Both the auxin-induced growth process and the uptake of ions are suppressed by metabolic inhibitors. Progress curves for the uptake of 2,4-D in the presence of KCN are shown in figure 6-A. For this experiment, coleoptile sections were treated for 25 minutes in .0025 *M* potassium maleate buffer solutions containing varied concentrations of KCN. After the period of preincubation, the sections were transferred to fresh solutions of similar compositions but containing in addition 0.5 mg/l of labeled 2,4-D. KCN in a concentration of 10^{-3} *M* decreases the amount of 2,4-D taken up by the initial process by about 45 per cent and inhibits the rate of continuing 2,4-D uptake by approximately 53 per cent. Higher concentrations of KCN, for example, 10^{-2} *M*, decrease 2,4-D uptake even more but are toxic to the tissue. It appears then that the continuing uptake of 2,4-D is dependent on metabolic energy. The initial uptake in the presence of 10^{-3} *M* KCN corresponds to that expected
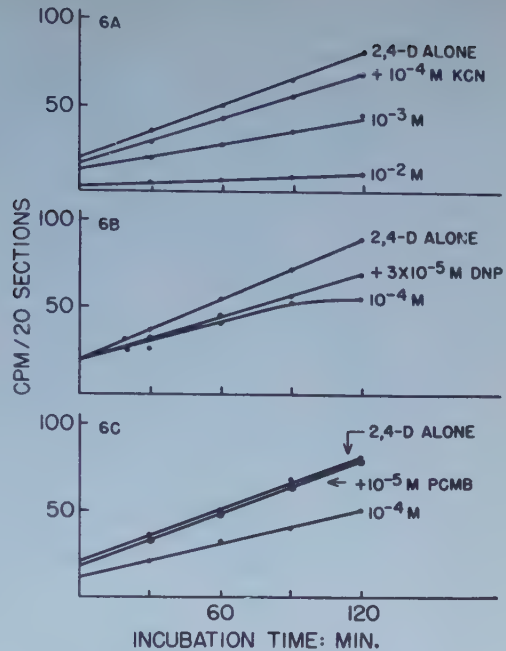
Figure 6. *Uptake of C^{14} carboxyl labeled 2,4-D (0.5 mg/l) as affected by varied metabolic inhibitors.*
Figure 6-A. *Uptake of 2,4-D in the presence or absence of KCN in varied concentrations.*
Figure 6-B. *Uptake of 2,4-D in the presence or absence of 2,4-dinitrophenol (DNP) in varied concentrations.*
Figure 6-C. *Uptake of 2,4-D in the presence or absence of parachloromercuribenzoate (PCMB) in varied concentrations.*

for diffusion into 0.15 of the tissue fresh weight. It is possible that KCN in this concentration may repress the exchangeable binding of 2,4-D, although further study of this matter has not been attempted.

Both the uptake of salts and auxin-induced growth are inhibited by the phosphorylative uncoupling agent 2,4-dinitrophenol (DNP). The effects of this inhibitor on 2,4-D uptake are shown in figure 6-B. The sections used in these experiments were pretreated for one hour in buffer solutions with or without DNP before being transferred to solutions containing labeled 2,4-D 0.5 mg/l. Initial uptake of 2,4-D is little affected by DNP, even at concentrations as high as 10^{-4} *M*. The presence of DNP does not alter the amount of 2,4-D which is lost or exchanged when the 2,4-D-treated tissue is transferred to solution containing unlabeled 2,4-D. Continued uptake of 2,4-D, on the other hand, is greatly reduced in the presence of 10^{-4} *M* DNP. A DNP concentration of 3×10^{-5} *M* has been found to inhibit the growth of Avena coleoptiles by 96 per cent over a 2 hour growth period. This concentration of DNP does not inhibit initial uptake at all but does inhibit the continuing uptake of 2,4-D by 33 per cent.

The sufhydryl reagent parachloromercuribenzoate (PCMB) is an inhibitor of auxin-induced coleoptile section growth (11). Figure 6-C presents data from experiments on the effect of PCMB on rate of 2,4-D uptake by sec-
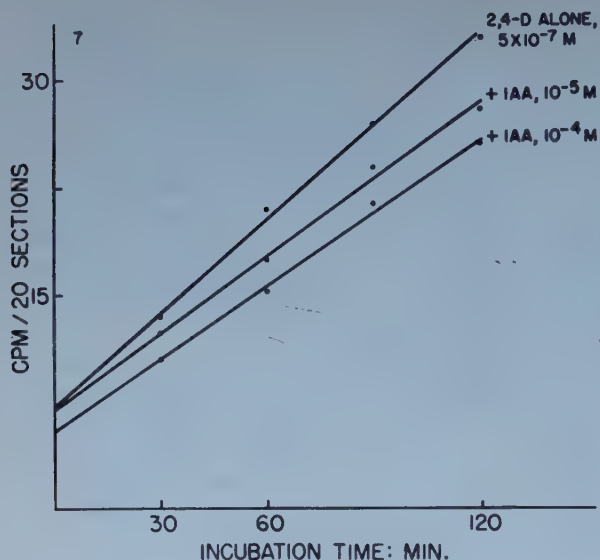
Figure 7. *Uptake of* C^{14} *carboxyl labeled 2,4-D by Avena coleoptile sections in the presence or absence of varied concentrations of indoleacetic acid* (IAA). *Concentration of labeled 2,4-D 0.1 mg./l* $(5 \times 10^{-7}\ M)$.

tions. All sections were pretreated for one hour in buffer with or without PCMB in various concentrations and were then transferred to solutions containing the inhibitor and labeled 2,4-D (0.5 mg/l). Lower concentrations of PCMB, as $10^{-5}\ M$ and $10^{-6}\ M$, have essentially no effect on either type of 2,4-D uptake. However, in the presence of $10^{-4}\ M$ PCMB, the continuing uptake of 2,4-D in reduced by 50 per cent and the initial uptake is decreased to essentially that expected for diffusion alone.

Competition Effects: The initial uptake and accumulation of a cation by plant tissue may be competitively inhibited by an appropriate second cation (Epstein and Hagen, 4). An auxin such as 2,4-D may also be competitively inhibited in its growth-promoting function by a second auxin, such as IAA (7). The continued uptake of 2,4-D into the coleoptile section is however, markedly inhibited only by exceedingly high concentrations of IAA.

For these experiments sections were placed in mixtures of labeled 2,4-D and unlabeled IAA. In order to increase any possible competitive inhibition of 2,4-D uptake by IAA, the 2,4-D concentration was reduced to 0.1 mg/l $(5 \times 10^{-7}\ M)$. The data of figure 7 show that IAA in a concentration of $10^{-5}\ M$ exerts little effect on rate of 2,4-D uptake. IAA in a concentration of $10^{-4}\ M$ (200 fold greater than the 2,4-D concentration), slightly inhibits both initial uptake and continued absorption of 2,4-D. No increased inhibition of 2,4-D uptake was obtained if the tissue was preincubated with IAA for 30 minutes.
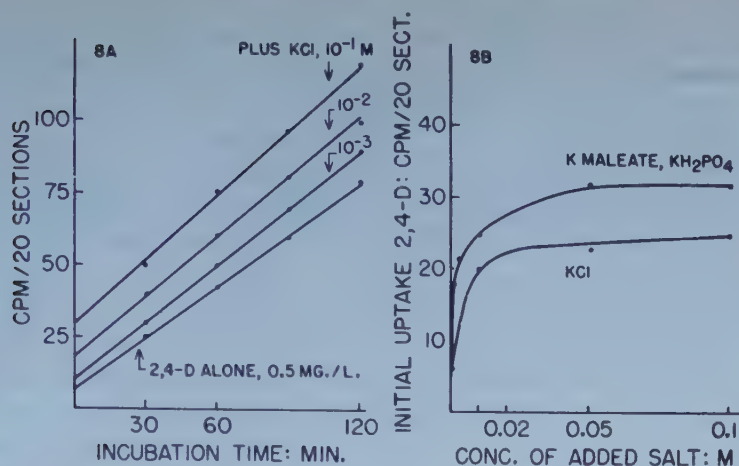
Figure 8. *Uptake of* C^{14} *carboxyl labeled 2,4-D (0.5 mg/l) as affected by the presence or absence of varied salts in the external medium.*

Figure 8-A. *Progress curves for uptake of 2,4-D in the presence of varied concentrations of KCl.*

Figure 8-B. *Initial uptake of 2,4-D as a function of concentration of added salts.*

It has been shown in Figure 3-B above that IAA in a concentration of 5×10^{-4} M is capable of releasing exchangeably bound labeled 2,4-D just as in an excess of unlabeled 2,4-D. It has also been shown that IAA in this concentration lowers the amount of 2,4-D taken up by the initial process to the level of that entering by diffusion alone. IAA does apparently compete with 2,4-D for exchangeable binding sites within the plant but does so relatively ineffectively.

Effect of Salts on 2,4-D Uptake: It has been established by earlier workers (3, 12) that the auxin-induced growth of Avena coleoptile sections is influenced by the ionic composition of the medium. Potassium salts in particular enhance the growth promoting effect of auxin. The uptake of 2,4-D is also influenced by the ionic composition of the medium. That this is so is shown by the data of figure 8-A, which concern uptake of 2,4-D in media containing various concentrations of KCl. For this experiment the 2,4-D stock solution was not neutralized and potassium ions were supplied only by the tissue and by the KCl added to the medium. It is clear from the data of figure 8-A that the amount of 2,4-D taken up by the initial process is increased in the presence of KCl. This KCl effect is saturated at about .02 M (figure 8-B). The rate of continuing uptake of 2,4-D is little affected by the presence of KCl.

Other cations including Na, Ca, and Mg can replace potassium in increas-

Table 6. *Loss of labeled 2,4-D by coleoptile sections to media of differing ionic composi-*
tions. Tissue transferred to such media after 30 min. incubation in labeled 2,4-D (gas
flow counting).

Concentration of 2,4-D in incubation medium in mg/l	CPM/20 sections			
	2,4-D taken up by initial process	2,4-D lost in		
		Distilled water	Maleate buffer 0.02 M	Maleate buffer 0.0025 M + 2,4-D, 20 mg/l
0.50	101	102	67	95
0.10	32	30	21	31

ing the amount of initial uptake, and other anions can replace chloride in
this function. The data of figure 8-B show that the initial uptake of 2,4-D
is particularly increased by the presence of KH_2PO_4 or of K-maleate and
that the effect of these salts approaches saturation only at approximately
.02 M. It has also been found that although the amount of initial uptake
of 2,4-D is controlled in part by the ionic composition of the media, the
dependence of the initial uptake of 2,4-D on external 2,4-D concentration is
quite similar under the various ionic conditions.

The ionic nature of the external medium influences not only the uptake
of 2,4-D by coleoptile tissue but also the amount of 2,4-D lost when tissue
is transferred to 2,4-D-free solution. For the experiments of table 4, sections
were incubated for 30 minutes in medium containing 0.0025 M maleate
buffer and labeled 2,4-D. After 30 minutes sections were transferred either to
distilled water or to 0.02 M maleate buffer. The total loss of labeled 2,4-D
was measured after 60 minutes. More 2,4-D was lost to distilled water than
to buffer. The 2,4-D lost to distilled water closely approximates that taken
up in the initial rapid uptake. That lost to buffer, on the contrary, makes
up only a portion of the total thus taken up. As noted above and demonstrat-
ed again in table 4, the presence of an excess of unlabeled 2,4-D in the
external buffer solution increases the loss of labeled 2,4-D from the tissue.
In fact, the total loss of labeled 2,4-D to distilled water equals that to high
concentration of unlabeled material, and both equal the total taken up by
the initial process. This may signify that the exchangeably bound 2,4-D,
is lost to water but not to buffer, although it may also be lost if it is
exchanged for unlabeled 2,4-D.

Discussion

Previous work on the uptake of auxin by plant tissues has been reported
by Albaum *et al.* (1), Sutter (10), and by Rheinhold (8). In the latter two
cases the uptake of auxin was estimated by chemical determination of

indoleacetic acid disappearance from the medium. The sensitivity of the methods used limited the concentration range of external auxin concentration to 10^{-4} M and greater. In neither case was it possible to follow auxin uptake from external auxin concentrations in the physiological range which promotes growth of Avena coleoptile sections, pea stems, and other tissues. Sutter's conclusion, shared by Albaum *et al.* (1), that IAA tends to attain a diffusion equilibrium between external solution and plant tissue is not confirmed in the present work, which shows clearly that the auxin 2,4-D is accumulated. The present experiments indicate, in agreement with the conclusions of Reinhold (8), that the uptake of auxin by plant tissue consists of at least two separable components. An initial rapid absorption is consummated in the case of the Avena coleoptile section in 30 minutes or less. With the carrot roots studied by Reinhold this initial rapid uptake is consummated within 4 hours. In both cases the initial uptake tends to be less sensitive to metabolic inhibitors than is the subsequent continuing uptake of auxin. The progress curves for IAA uptake by cucurbit hypocotyl sections presented by Sutter also show a clear separation into an initial process consummated in approximately 1.5 hours and a slower continuing uptake.

The present work indicates however that the initial rapid uptake of an auxin consists of 2 separable components, and that in total at least 3 kinds of uptake of 2,4-D by the coleoptile appear to be involved. These are respectively: (1) a diffusion into an accessible fraction of the tissue, (2) an exchangable binding, and (3) a continuing uptake. These processes have the following characteristics.

1. Diffusion into the tissue.
    a. Uptake goes to completion within 30 minutes.
    b. 2,4-D taken up by tissue is free to diffuse out again.
    c. Inward diffusion is not influenced by even large excesses (1000 fold) of IAA.
    d. Not affected by metabolic inhibitors.

2. Exchangeable binding in the tissue.
    a. Uptake goes to completion within 30 minutes.
    b. 2,4-D once bound cannot leach out again (to buffered solution) but can be released by excess unlabeled 2,4-D or by IAA. The exchangeably bound 2,4-D also appears to be hydrolyzable and is releazed to water in the absence or buffer or other salts.
    c. Uptake is suppressed in the presence of a sufficient excess of IAA.
    d. Uptake is not inhibited by DNP.

3. Continued uptake.
    a. Accumulation goes on steadily over a period of hours.

    b. 2,4-D taken into tissue is not diffusible or exchangeable.

    c. Rate is inhibited by sufficient excess of IAA.

    d. Rate is inhibited by metabolic inhibitors including DNP.

What now is the relation of the uptake of auxin by the Avena coleoptile section to the auxin-induced growth process in this tissue? It is clear at once that the growth rate of the tissue cannot be determined by its total auxin content. Auxin uptake by the coleoptile section continues over a period of hours, during which time the growth rate remains constant. On the other hand, the section is immediately responsive to changes in the external concentration of auxin, as has been shown by McRae, Foster, and Bonner (7), and others. In a sense then it appears to be the external auxin concentration rather than the total internal concentration which determines the growth rate of the coleoptile section. It is possible that the bulk of the auxin taken up by the tissue merely accumulates in the vacuole, as is the case with inorganic ions.

The action of auxin in initiating the growth process may be mediated through the consummation of an attachment between auxin and an appropriate receptor within the plant (5). We may therefore ask whether the exchangeable binding of auxin to coleoptile tissue as observed in the present work may be a reflection of the complex formation between auxin and plant which has been proposed on purely kinetic grounds. It is not obvious that this is a correct conclusion. The initial exchangeable binding of 2,4-D as measured in the present experiments is little affected by the presence or absence of a second auxin (IAA) present in concentrations sufficient to completely dominate the growth process (7).

The continuing uptake of 2,4-D similarly cannot readily be identified with the process of auxin-induced growth. It is true that the continuing uptake of 2,4-D by section tissue is metabolically powered as is the auxin-induced growth of the section. The same is true of uptake of ions in general and is not therefore unique to the uptake of 2,4-D. In addition, the continuing uptake of 2,4-D by Avena section tissue is only slightly depressed by the presence of a large excess of a second auxin, IAA, in the external solution. In this relationship the uptake of 2,4-D by the continuing process is again quite different from the auxin relations of the growth process.

Summary

1. The uptake of auxin by Avena coleoptile sections has been studied by the use of C^{14} carboxyl labeled 2,4-D of high specific activity. The material remains in the tissue in largely unaltered form for periods of at least 3 hours.

2. The uptake of 2,4-D by coleoptile tissue consists of three separable processes. The first, consummated in 20—30 minutes, resembles a diffusion into the tisssue in the sense that 2,4-D taken up by this process is readily leached to the outside solution. The second also a rapid process resembles a binding, since the 2,4-D bound can be removed by exchange for unlabeled 2,4-D. The third component of 2,4-D uptake is a continuing absorption, maintained at a steady rate for several hours. 2,4-D may be accumulated by the tissue to a concentration higher than the external concentration.

3. The rate of continuing uptake of 2,4-D is inhibited by low temperature, by the metabolic inhibitors KCN. 2,4-dinitrophenol, and p-chloromercuribenzoate. In general. the initial diffusion is little inhibited by these same inhibitors.

4. The rapid initial uptake of 2,4-D by Avena section tissue is increased in amount in the presence of salts such as KCl, K maleate or KH_2PO_4. This effect appears to be primarily upon the exchangeably bound component.

5. Both the exchangeable binding and the continuing uptake of 2,4-D are inhibited by the presence of a high concentration of a second auxin, IAA. Uptake of 2,4-D is less influenced by these substances than is the 2,4-D induced growth of the coleoptile.

References

1. Albaum, H. G., Kaiser, S. & Nestler, H. A.: The relation of hydrogen ion concentration to the penetration of 3-indoleacetic acid into Nitella cells. — Amer. Jour. Bot. 24: 513. 1937.
2. Bonner, J. & Thimann, K. V.: Studies on the growth hormone of plants. 7. The fate of growth substance in the plant and the nature of the growth process. — Jour. Gen. Physiol. 18: 649. 1935.
3. Cooil. B.: Relationships of certain nutrients, metabolites and inhibitors to growth in the Avena coleoptile. — Plant Physiol. 27: 49. 1952.
4. Epstein, E. & Hagen, C. E.: A kinetic study of the absorption of alkali cations by barley roots. — Plant Physiol. 27: 457. 1952.
5. Foster, R. J., McRae, D. H. & Bonner, J.: Auxin-induced growth inhibition a natural consequence of two point attachment. — Proc. Nat. Acad. Sci. 38: 1014. 1952.
6. McRae. D. H. & Bonner, J.: Chemical structure and antiauxin activity. — Physiol. Plant. 6: 485. 1953.
7. McRae, D. H., Foster, R. J. & Bonner, J.: Kinetics of auxin interaction. — Plant Physiol. 28: 343. 1953.
8. Reinhold, Leonora: The uptake of indole-3-acetic acid by pea epicotyl segments and carrot discs. — New Phytologist 53: 218. 1954.
9. Steward, F. C. & Harrison, J. A.: The absorption and accumulation of salts by living plant cells. 9. The absorption of rubidium bromide by potato discs. — Ann. Bot. N.S. 3: 427. 1939.

10. Sutter, Erica: Die chemische Bestimmung des Heteroauxins und Versuche über seine Aufnahme durch die Pflanze. — Ber. d. Schweiz. bot. Ges. 54: 197. 1944.

11. Thimann, K. V. & Bonner, W. D.: Growth and inhibition of isolated plant parts. 2. The action of several enzyme inhibitors on the growth of the Avena coleoptile and on Pisum internodes. — Amer. Jour. Bot. 36: 214. 1949.

12. — & Schneider, C. L.: The role of salts, hydrogen ion concentration and agar in the response of Avena coleoptile to auxin. — Amer. Jour. Bot. 25: 270. 1938.

The "Rhizosphere Effect" of Graminaceous Plants in Virgin Soils

II. Nutritional Characteristics of Non-sporogenous Bacteria Associated with the Roots

By

Helge Gyllenberg

Department of Microbiology, University of Helsinki, Finland
(Received November 18, 1955)

In a previous paper (Gyllenberg, 1955) quantitative data concerning the "rhizosphere effect" of graminaceous plants in virgin soils were reported. Evidence was cited to show that the "rhizosphere effect" under the conditions investigated was most distinctly confined to non-sporogenous bacteria. In this second report some properties of these bacteria will be described. In this connection particular attention is paid to the nutritional characteristics which may provide some information to explain the close dependence of these bacteria on rhizosphere conditions.

Several reports have been published on the nutritional features of soil bacteria, and methods have been developed for a systematical study of soil bacteria in this respect (e.g. West and Lochhead, 1940; Lochhead and Chase, 1943; Lochhead and Thexton, 1947, 1952; Katznelson and Richardson, 1948; Taylor, 1951; Lochhead, 1952). The results in general show that the development of bacteria having simple nutritional requirements (capable of growth with inorganic nitrogen and without an addition of B-vitamins) and of those dependent on amino acids is favoured in the rhizosphere. This provides indirect evidence to show that amino acids are more easily available in the rhizosphere than in "non-rhizosphere" soil, which obviously is due to amino acid secretion by the plant roots. As regards certain culture plants (e.g. flax, tobacco, and strawberry which have been extensively investigated) the

Table 1. *The "systematic" grouping of the cultures investigated.*

Desig-nation of group	No. of cult-ures	Morphology of cells	Gram stain	Pigments	Growth on nutri-ent agar
I	12	Short rods 0.5 by 1—4 μ	Negative	Agar media turn brown or greenish	Good
II	10	Usually very short, nearly coccoid rods	Negative	Agar media remain un-coloured, but yellow pigments common	Good
III:1	24	Very variable, long filaments, short rods, and coccoid forms	Generally positive, some strains variable	Yellow or pale brown pigments, in addition to unpigmented strains	Very poor
III:2	14	Irregular forms, but filaments rare	Positive to variable	Lemon yellow, red, or unpigmented	Good

"rhizosphere effect" may thus, at least partly, arise from more favourable nutritional conditions close to the plant roots than at a distance from them. So far as virgin soils are concerned, there is no corresponding information available. Differences between cropped and virgin soils (especially forest soils) in the distribution of essential organic nutrients for the soil bacteria are. however, likely to occur, as emphasized by Schmidt and Starkey (1951) re-garding the B-group vitamins.

Results

General characteristics of the cultures. 177 cultures, isolated from rhizo-sphere samples during the quantitative investigations (Gyllenberg, 1955), were selected for preliminary routine characterization. On the basis of these tests, the collection was limited to 60 cultures only. Hereby care was taken to include in the final collection all different morphological and physiological types recognized.

More extensive characterization tests of each individual culture revealed that the isolates fell into the main "systematic" groups indicated in Table 1. It was not tried to obtain a definite determination of the genera and species of the cultures, but in general it seemed that the group I strains most closely were related to Pseudomonas. the group II strains to Achromobacter and Flavobacterium, and the group III strains to Corynebacterium or similar types (e.g. Bacterium globiforme).

Nutritional characteristics of the cultures. The method of nutritional classi-fication of soil bacteria developed by Lochhead and Chase (1943) is based on determination of the growth figures for each isolate in 7 different media.

Table 2. *The media employed for the nutritional testing of the cultures.*

Designation of medium	Additions		
	Basal constituents	Vitamin solution	»Casamino acids»
Ba	+	—	—
BaV	+	+	—
Aa	+	---	+
AV	+	+	+

The nutritional group into which a culture falls then depends on the medium first showing maximal growth when the media are compared in order of increasing complexity. Taylor (1951) has proposed the use of 5 media only, and some other modifications of the method. Although the purpose of the present investigation was not to obtain a definite "nutritional classification" of the cultures, but rather to determine the nutritional conditions where each culture developed growth, a method similar in principle to those of Lochhead and Chase and Taylor was used. Accordingly, a grouping on the basis of nutritional characteristics could be obtained, and, as will be shown, this grouping proved rather satisfactory.

The nutritional testing of each strain was carried out in the following way: The basal medium contained 0.8 g KNO_3, 0.8 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7 M_2O$, 0.2 g NaCl, 0.02 g $FeCl_3$, and 2 g glucose in 1000 ml of distilled water (pH 6.8). By addition of amino acids (Vitamin-free Casamino acids, Difco) and/or B-vitamins (a solution containing thiamin, biotin, pantothenic acid, folic acid, p-aminobenzoic acid, and Vitamin B_{12}) 3 additional media were set up as indicated in Table 2. These media were inoculated with washed cells suspended in sterile saline. Incubation was carried out at room temperature for 8 days. A measure of growth was obtained turbidimetrically (Beckman Spectrophotometer, wave length 650 μ). In some preliminary experiments visual scoring was employed.

This arrangement of testing is much simpler than those of Lochhead and Chase (1943) and Taylor (1951). The most significant modification was that media containing yeast and/or soil extracts were excluded from the scheme. This seemed advisable since preliminary experiments with several cultures showed no considerable increase in growth figures upon addition of these extracts. Moreover, it may be emphasized that vitamin B_{12} was included in the vitamin addition, and that, in accordance with the proposal of Taylor (1951), "casamino acids" were added as the source of amino acids. Although the author's method was satisfactory for its limited purpose, there is no evidence of its general applicability.

According to the author's method each culture may show one of the following nutritional characteristic:

(a) the culture requires no organic nitrogen and no B-vitamins for growth.

(b) the culture requires no organic nitrogen but requires preformed B-vitamins for growth.

(c) the culture requires amino acids but no B-vitamins for growth.

(d) the culture requires both amino acids and B-vitamins for growth.

Table 3. *The nutritional characteristics of the "systematic" groups.*

Group	No. of cultures	Number of cultures					
		Simplest medium showing growth			Medium showing maximal growth		
		Ba	Aa	AV	Ba	Aa	AV
The whole collection ..	60	26	28	6	4	28	28
Group I	12	12				12	
Group II	10		10			10	
Group III	38	14	18	6	4	6	28
III: 1	24	4	16	4		4	20
III: 2	14	10	2	2	4	2	8

Accordingly, different "nutritional groups" were obtained, but rather 3 groups only, corresponding to alternatives a, c, and d (the group b seemed to have no practical significance). These groups were designed Ba (basal), Aa (amino acids), and AV (amino acids + vitamins), respectively cf. also Table 2).

Determination of the simplest medium showing definite growth seemed to provide information for assessing the nutritional conditions where different bacterial types are capable of development in soil. A grouping on the basis of the nutrient supplies required for maximal growth, on the other hand, revealed distinctly different results but a comparison of the results obtained with both methods was a rather valuable tool in the characterization of the nutrional features of individual cultures and different "systematic" groups. Table 3 presents the results as regards the whole collection and the "systematic" groups described above (cf. Table 1).

From Table 3 it is evident that although a considerable number of cultures were capable of growth in the basal medium, the growth of most of them was greatly stimulated in the presence of amino acids and/or B-vitamins. A clearer picture of this fact is obtained when the nutritional features of the different "systematic" groups are compared. The results show that all the Gram-negative short rod cultures (groups I and II) were independent of B-vitamins. The group I cultures developed readily in the basal medium, but maximal growth figures were obtained in the presence of amino acids. The group II cultures again required amino acids as essential growth factors. The cultures related to corynebacteria (group III) showed more heterogenous characteristics. B-vitamins were required for maximal growth by 28 cultures (out of 38), obviously also amino acids by several cultures (only 4 out of 38 cultures developed maximal growth in the basal medium). The III: 1 cultures seemed to require more complex nutrient supplies than the III: 2 cultures. It is especially interesting to note that each "systematic" group showed a characteristic

Table 4. *The average growth figures with different sources of nitrogen* (density readings).

Group	Nitrogen source		
	KNO_3	$(NH_4)_2SO_4$	»Casamino acids»
I	0.125	0.125	0.205
II	0.025	0.060	0.145
III: 1	0.010	0.030	0.065
III: 2	0.040	0.055	0.075

nutritional behaviour which fact confirms the validity of the systematic grouping carried out.

The tests described above already showed that amino acids may have a distinct promoting effect on the development of the groups of soil bacteria investigated. Taylor (1951) states, however, that ammonium salts replace amino acids as nitrogen source for an abundant number of soil bacteria, and that cultures which fail to grow on KNO_3 rather are incapable of utilizing this nitrogen source than definitely dependent on organic nitrogen. To prove this consideration an experiment was set up employing media containing B-vitamins and KNO_3, $(NH_4)_2SO_4$, and casamino acids, respectively, as the nitrogen source. The results of this experiment, given in Table 4, indicate that, except the group I cultures, ammonium sulphate gave rise to some growth promotion as compared with the growth figures in the KNO_3 medium, but also that the ammonium salt did not produce maximal growth figures. This held true as regards each individual culture tested. Accordingly, it can be concluded that amino acids either effect a considerable growth promotion or are essential growth factors for most types of non-sporogenous bacteria occurring in the rhizosphere of graminaceous plants in virgin soils.

The effect of individual amino acids was not investigated in this connection, but the effect of cystine, and some mixtures of amino acids (containing cystine, aspartic acid, alanine, proline, lysine, leucine, glycine, glutamic acid, and arginine in different combinations) were compared with the effect of "casamino acids". These experiments revealed that cultures of group I were stimulated by any addition of amino nitrogen, but always most strongly so by "casamino acids". Cystine alone showed no effect on cultures of group II, but considerable growth took place when all the amino acids listed above were added. However, maximal growth figures were obtained only by addition of "casamino acids". As regards the cultures of group III again, the nutritional requirements of some strains were satisfied already by an addition of cystine only, with most other strains an addition of some individual amino acids was sufficient to produce maximal growth, and a few strains only required "casamino acids".

Table 5. *The dependence of the vitamin-requiring cultures on supplies of different vitamins* (28 cultures were tested).

Medium	Average of relative growth figures	No. of cultures showing decrease in growth
AV	100	
AV, without thiamin	77	24
AV, without pantoth. acid	82	15
AV, without biotin	62	28
AV, without folic acid	78	14
AV, without vitamin B_{12}	89	10
AV, without p-aminobenzoic acid ..	85	23

As shown in Table 3, B-vitamins were required for maximal growth by 28 out of the 60 cultures studied. In Table 5 results are presented as regards the requirement of the different individual vitamins supplied with the vitamin solution added to medium AV. From Table 5 it is obvious that all the cultures showing dependence on B-vitamins required biotin. The major part of the cultures were dependent on p-amonibenzoic acid and thiamin also. About 50 per cent of the cultures required pantothenic acid and/or folic acid in addition. 10 cultures out of 28 showed requirement of vitamin B_{12}.

In this connection it must be noted, that although considerable growth figures were obtained in the vitamin-free media Ba and Aa, and also in the medium AV in the absence of a certain individual vitamin, the vitamins in question can be considered as essential nutrients for most of the cultures of group III. This is evident from the fact that the growth figures could be gradually brought down to insignificant levels when a culture was kept in a vitamin-free medium for a number of successive passages. This gradual development of the requirement of preformed vitamins is not, however, due to variation induced during laboratory cultivation since the ability to grow in vitamin-free media reappeared after one or two passages in media richly supplied with B-vitamins. Preliminary experiments showed that in a vitamin-free medium only poorly supplied with other nutrients growth developed through a higher number of passages than when a vitamin-free, but otherwise sufficient medium was used. In the latter medium good development of growth took place in the first passages, but the growth figures dropped down upon a few transfers. In the poor medium again, the growth figures were initially low, but this level was maintained rather unchanged for several successive passages. This may show that these bacteria possess an ability to "store up" certain essential substances in their cells when the external supply of preformed vitamins is rich. By intense growth in a vitamin-free medium this "reserve" is rapidly used up, while it suffices for a much longer time when the growth proceeds slightly.

The "rhizosphere effect". The results presented above provide some matter for discussing the "rhizosphere effect". As shown in Table 3 34 cultures out of 60 failed to grow in the absence of amino acids, and of the remaining 26 cultures, the growth of 22 cultures was distinctly promoted by amino acids. Al-

though the effect of individual amino acids remained unclear in this connection, it can be concluded that most groups of "rhizosphere bacteria" increase in numbers when furnished with amino nitrogen. This effect is considerably stronger when a many-sided combination of amino acids is available.

Free amino acids may be excreted into the soil by plant roots, or may arise from the decomposition of plant residue proteins. It seems, however, that the "amino acid effect" on "rhizosphere bacteria" is rather due to root secretion than to the activity of proteolytic bacteria. Among the 177 "rhizosphere cultures" initially investigated, only a few showed pronounced proteolytic activity, and, as already stated, only 4 cultures out of the 60 in the final collection developed maximal growth in the absence of amino acids.

On the other hand, the growth promotion of bacteria requiring preformed vitamins cannot be explained as caused only by root secretion of essential or stimulatory nutrients. It has been shown, however, that soil bacteria which are independent on supplies of preformed vitamins produce vitamins in excess of their own requirements, and that vitamins then can be recovered outside the bacterial cells (e.g. Starkey. 1944). Accordingly, it could be supposed that the cultures of groups I and II, which showed no vitamin requirement, would be capable of vitamin synthesis, and thus also of promoting the growth of vitamin-requiring cultures (group III).

To prove the assumptions of a direct "rhizosphere effect" on amino acid dependent cultures, and of the vitamin synthesis by vitamin independent cultures, a number of experiments was performed using *Lolium multiflorum* as test plant.

Seeds of Lolium multiflorum were germinated on water agar. The plates were time by time exposed to ultraviolet radiation in order to avoid mold infection and to decrease the number of contaminants in the seeds. After germination some 20 germinated seeds were transferred to 50 ml Erlenmeyer flasks containing 10 ml of medium Ba. Proper light conditions were arranged, and a number of the flasks was then inoculated with cultures belonging to group II (cultures requiring amino acids, but no B-vitamins). Controls inoculated with group II cultures, but containing no growing plants were also set up. After 10—14 days, during which time good development of L. multiflorum was recognized, the numbers of bacteria in the growth solutions were counted microscopically, and by dilution methods.

It was then found that a rather poor growth of bacteria took place in the uninoculated flasks with L. multiflorum. The bacterial flora seemed to consist of a chain-forming coccus, obviously a contaminant of the seeds not destroyed by the UV-radiation. In the inoculated controls without L. multiflorum, the development of the group II cultures was very poor as expected, but in the inoculated flasks with L. multiflorum 60—100 times higher figures were found. Spreadings on agar and reisolation of the inoculated organisms

Table 6. *The effect of Lolium multiflorum on the growth of amino acid-requiring cultures in an amino acid-free medium* (Ba). *The figures are numbers of bacterial cells (millions per ml).*

Inoculum	With L. multiflorum	Without L. multiflorum
None	0.6	—
Culture 217	243.2	3.4
Culture 1003	127.1	2.4
Culture 1017	351.4	3.8

from these flasks showed no considerable development of contaminants. The results of the microscopic counts are given in Table 6.

The growth solutions from certain flasks in the previous experiment were collected and filtered through a bacterial filter (Membranfilter). 0.2 ml of these filtrates were added to 9.8 ml of medium Aa in test tubes. 2 cultures belonging to group III (requiring both amino acids and B-vitamins) were then tested for growth in the solutions obtained. The media Aa and AV were used as controls. The results are given in Table 7.

The results of the experiments described (Tables 6 and 7) clearly show that the group II cultures developed considerable growth in the presence of Lolium multiflorum, whereas they did not grow at all in the same amino acid-free medium in the absence of the growing plant. The group III cultures again, when cultivated in a vitamin-free medium, were stimulated by addition of cell-free filtrates of solutions where the group II cultures had grown. L. multiflorum did not effect growth promotion of the group III cultures. The growth figures in the latter experiment remained, however, lower than those effected in the ordinary vitamin-containing medium (AV).

Discussion

The present investigation concerns the nutritional requirements of some types of non-sporogenous bacteria from virgin soils in relation to the "rhizosphere effect" of graminaceous plants. Although it must be considered that several other factors (e.g. pH, soil structure, surface phenomena etc.) are involved in the "rhizosphere effect", the results obtained evidently show that the stimulation of soil bacteria in the rhizosphere to a significant degree depends on favourable nutrient supplies. Amino acids arising from root secretion highly promote the development of those types of soil bacteria which do not require other preformed organic nutrients (i.e. vitamins). These bacteria produce vitamins in excess of their own requirements, and it seems that this excess of vitamins is secreted outside the bacterial cells. Other types of bac-

Table 7. *The effect of cell-free filtrates of group II cultures* (grown with and without L. multiflorum) *on the growth of vitamin-requiring strains in a vitamin-free medium* (Aa). The figures are density readings. In the brackets relative growth figures are given.

Inoculum	Medium or group II culture grown	Experiment	
		With L. multiflorum	Without L. multiflorum
420	Aa — control	0.035 (54)	0.035 (54)
	culture 217	0.055 (85)	0.040 (61)
	culture 1003	0.045 (69)	0.035 (54)
	culture 1017	0.055 (85)	0.035 (54)
	AV — control		0.065 (100)
1211	Aa — control	0.035 (58)	0.030 (50)
	culture 217	0.045 (75)	0.030 (50)
	culture 1003	0.040 (67)	0.030 (50)
	culture 1017	0.050 (83)	0.035 (58)
	AV — control		0.060 (100)

teria requiring preformed vitamins are, in turn, stimulated by the vitamin supply introduced in this way.

As to the role of amino acids as stimulatory or essential nutrients for "rhizosphere bacteria" the author's results are in good agreement with those reported by other authors (West and Lochhead, 1940; Lochhead and Thexton, 1947; Katznelson and Richardson, 1948). Lochhead and Thexton (1947) reported, however, that bacteria requiring preformed vitamins are relatively more numerous in "non-rhizosphere" soil than in the rhizosphere, and that the "rhizosphere effect", therefore, cannot be considered confined to these types of soil bacteria. In this connection the data presented by Schmidt and Starkey (1951) as regards the riboflavin contents of the humus layers of forest and cropped soils must be considered. These authors reported that the amounts of riboflavin are up to 50 times higher in forest soils than in agricultural soils, and they also assumed that a corresponding difference probably occurs as regards other B-vitamins. It can be assumed, therefore, that the distribution of vitamin-requiring bacteria in cropped and in forest soils is decisively different.

Lochhead and Thexton (1952) have cited evidence to show that vitamin B_{12} produced by some types of soil bacteria is a utilizable supply of this substance for bacteria requiring it. This finding as well as the data reported by Starkey (1944) agree with the results obtained in the present study as regards the close nutritional associations which occur between different groups of soil bacteria.

The phenomenon observed during the course of the present investigation, that the growth of vitamin-requiring bacteria proceeds through several generations without external vitamin supplies is noteworthy. This behaviour,

when investigated in more detail than was possible in the present study, probably would reveal new features of the nutrition of bacteria under the conditions prevailing in soil. The finding that the dependence on external vitamin supplies is lower during a low growth rate than during vigorous growth is especially interesting, and this property may be important for the maintenance of the life functions of these bacteria in soil. As considered by Schmidt and Starkey (1951), the rate of microbial synthesis of B-vitamins exceeds that of destruction during periods of rapid decomposition of organic matter, and accompanying strong microbial activity in the soil. During periods of low microbial activity again, there may be no external B-vitamins available. It can be supposed, therefore, that the relative numbers of vitamin-requiring types are maintained rather unchanged irrespective of prevailing soil conditions.

Summary

60 cultures of non-sporogenous "rhizosphere bacteria" isolated from the rhizosphere of graminaceous plants in virgin soils may be grouped as 3 main groups:

(I) Gram-negative short rod cultures, capable of growth with KNO_3 as nitrogen source, but distinctly stimulated upon addition of amino acids. Require no B-vitamins.

(II) Gram-negative, short rod cultures, failing to grow with KNO_3 as nitrogen source; requiring amino acids, but no B-vitamins.

(III) Gram-positive to Gram-variable cultures, showing irregular morphology; most cultures requiring both amino acids and B-vitamins.

It was found that except 4 cultures of group III all the cultures either required amino acids or were distinctly stimulated to more vigorous growth upon addition of amino acids. Experiments with *Lolium multiflorum* showed that a direct "rhizosphere effect" on cultures independent of B-vitamins (group I and II) may arise from secretion of amino acids by plant roots. The soil bacteria whose growth is promoted in this way are capable of vitamin synthesis, which, in turn, gives rise to a growth promotion of bacteria requiring preformed vitamins (group III). Biotin was required by all the vitamin-dependent cultures, thiamin and/or p-aminobenzoic acid by most of them.

References

Gyllenberg, H.: The "rhizosphere effect" of graminaceous plants in virgin soils. — Physiol. Plant. 8: 644. 1955.

Katznelson, H. & Richardson, L. T.: Rhizosphere studies and associated microbiological phenomena in relation to strawberry root rot. — Sci. Agr. 28: 293. 1948.

Lochhead, A. G.: The nutritional classification of soil bacteria. — Proc. Soc. Appl. Bact-
    eriol. 15: 15. 1952.
—   & Chase, F. E.: Qualitative studies of soil microorganisms. V. Nutritional require-
    ments of the predominant bacterial flora. — Soil. Sci. 55: 185. 1943.
—   & Thexton, R. H.: Qualitative studies of soil microorganisms. VII. The "rhizosphere
    effect" in relation to the amino acid nutrition of bacteria. — Can. Jour. Res. C,
    25: 20. 1947.
—   &   —: Qualitative studies of soil microorganisms. X. Bacteria requiring vitamin B_{12} as
    growth factor. — J. Bact. 63: 219. 1952.
Schmidt, E. L. & Starkey, R. L.: Soil microorganisms and plant growth substances. II.
    Transformations of certain B-vitamins in soil. — Soil Sci. 71: 223. 1951.
Starkey, R. L.. Changes in the content of certain B-vitamins in organic materials decompos-
    ing under aerobic and anaerobic conditions. — Soil Sci. 57: 247. 1944.
Taylor, C. B.. The nutritional requirements of the predominant flora of the soil. — Proc.
    Soc. Appl. Bacteriol. 14: 101. 1951.
West, P. M. & Lochhead, A. G.: Qualitative studies of soil microorganisms. IV. The rhizo-
    sphere in relation to the nutritive requirements of soil bacteria. — Can. Jour. Res. C,
    18: 129. 1940.

Nitrogen Fixation by Strains of *Aerobacter aerogenes*

By

Vagn Jensen

Department of General Microbiology, Royal Veterinary and Agricultural
College, Copenhagen
(Received November 24, 1955)

Experiments

During 1954—1955 a study was made on the Azotobacter flora of Danish watercourses (Jensen, 1955). The Azotobacter strains were isolated by means of enrichment cultures with mannitol or ethanol as carbon source and subsequent plating on nitrogen-free glucose or ethanol agar.

During this search, colonies of a small, encapsulated, rod-shaped bacteria were observed repeatedly, growing rather vigorously on the mannitol and glucose media. Preliminary experiments with this organism showed a distinct nitrogen-fixing ability, and a few strains were isolated for further studies.

Description of the isolated strains. The following investigations comprise two strains, N_1, isolated from a brooklet in East Jutland, and N_5, isolated from a small river in North East Zealand. The two strains are very similar, both morphologically and physiologically.

In young cultures the cells are regularly rod-shaped, 1.4—1.9 μ × 0.7—0.9 μ. They are clearly gram-negative, and motility has not been observed. When grown on common nutrient agar the cells stain strongly and uniformly with the usual bacterial stains, and the encapsulation is little conspicuous or lacking. When grown on nitrogen-free media, however, the appearance of the cells is different. Preparations stained with crystal violet show a slightly stained protoplasm, containing a deeply stained, nucleus-like body, and surrounded by a strongly developed slime capsule, an appearance very similar to the "nuclear growth", described by Duguid (1948) for strains of Aerobacter aerogenes on nitrogen or phosphate-deficient media.

The organisms grow vigorously on nutrient agar form'ng a greyish white, moist, smooth, filiform growth. Isolated colonies are circular convex, ·v:th entire margin.

On nitrogen-free mannitol agar growth is also good, forming grey, more or less transparent, mucous colonies.

No liquefaction of gelatin is observed after two weeks incubation at $20°$.

Catalase is formed on nutrient agar.

Nitrates are reduced to nitrites.

Methyl red test is negative.

Voges-Proskauer test is positive (Barritt's modification).

Citrate test: N_5 grows vigorously on Simmon's citrate agar, N_1 only moderately to slightly, both show alkaline reaction.

Indol is formed from tryptophan broth by N_5 but not by N_1 (Ehrlich's reagent combined with the Gnezda oxalic acid test).

McConkey broth: Vigorous growth and formation of gas at $25°$, but no growth at $44°$.

Brilliant green bile: Strong growth and formation of gas at $25°$.

EMB agar: Vigorous growth. Colonies pink with no sign of metallic sheen.

Hydrogen sulphide is not produced from iron-peptone-agar.

Thermal death point: $55°$ (all cells killed in 5 min.).

Fermentation reactions: Glycerol, adonitol, sorbitol, mannitol, inositol, l-arabinose, l-xylose, glucose, fructose, d-mannose, d-galactose, saccharose, maltose, lactose, raffinose, and salicin are fermented with formation of acid and gas. Butanol, erythritol, and dulcitol are not fermented.

The two strains show in all essential characters good agreement with the usual descriptions of Aerobacter aerogenes (e.g. Parr, 1939, Bergey, 1948) and can with certainty be identified as belonging to this species.

Nutrient requirements. The organisms are able to fix elementary nitrogen, as will be shown in the following, but the growth is distinctly accelerated by the presence of combined nitrogen.

Furthermore the organisms grow well in a completely synthetic medium, when combined nitrogen is present (e.g. Fermi's solution), whereas they generally fail to grow in a synthetic nutrient solution without combined nitrogen. Growth and nitrogen fixation occurs only when a small amount of agar or — better — yeast extract is added to the medium.

It seems as if some growth factor, found in small amounts in agar and in greater amounts in yeast extract, is nescessary for nitrogen fixation, but the question is not yet settled, and requires further studies.

In the nitrogen fixation experiments, a medium of the following composition was used: Glucose or mannitol 10.0, K_2HPO_4 0.2, $MgSO_4$, $7 H_2O$ 0.2, NaCl 0.2, $CaSO_4$ $2 H_2O$ 0 1, $Fe_2(SO_4)_3$ 0.01, Na_2MoO_4, $2 H_2O$ 0.005, Yeast extract, Bacto 0.01 g. Dist. water 1000 ml.

A small amount of $CaCO_3$ was added to the culture flasks in order to neutralize the acid formed during the fermentation.

Influence of temperature on nitrogen fixation. The optimum temperature for growth in lactose broth, as measured by the amounts of gas developed in fermentation tubes, was found to lie between $25°$ and $35°$, a little higher for

Table 1. *Influence of temperature on nitrogen fixation.*

Temperature °C	Nitrogen fixed, mg pr flask	
	N_1	N_5
0	0	0
5	0.12	0.01
15	0.92	0.76
18	0.97	0.69
23	0.49	0.53
25	0.53	0.52
30	0 13	0.41
35	0.01	0.32
40	0	0.16
45	0	0

N_5 than for N_1. This is in agreement with the observations of previous investigators, who have found the optimum temperature of Aerobacter aerogenes generally about 30° (see e.g. Bergey, 1948).

The optimum temperature, as measured by the amount of nitrogen fixed in nitrogen-free media, however, lies at a considerably lower level, as indicated in Table 1.

The experiments, recorded in Table 1, were made in the following manner:
Erlenmeyer flasks (100 ml), each containing 20 ml nutrient solution with 1 % mannitol and 0.03 gr $CaCO_3$, were sterilized at 120° for 20 minutes, and inoculated with a cell suspension from a 1 day old culture, grown on nutrient agar. Control flasks were re-sterilized immediately after the inoculation, and the nitrogen content of cultures and control flasks determined after a 10 day incubation period at various temperatures. The control flasks contained about 0.2 mg nitrogen.

The nitrogen determinations were made by a semimicro Kjeldahl method, modification of Parnas and Wagner (Parnas, 1938), using a HgO-catalyst. It is possible by this procedure to determine the nitrogen content to about ± 0.01 mg.

All figures in this and the following tables represent averages of duplicate cultures.

The highest nitrogen yield was obtained at about 15°—18° for both strains. Minimum temperature for nitrogen fixation was found about 5° and maximum temperature about 35°—40°.

In a subsequent experiment (see Table 2) the ratio was determined between amount of carbohydrate decomposed and amount of nitrogen fixed. The procedure was as follows:

Erlenmeyer flasks (100 ml), each containing 20 ml nutrient solution without carbon source and 0.2 gr $CaCO_3$, were sterilized at 120° for 20 minutes. 5 ml 5 % glucose solution (Bacto), sterilized by filtration, was added aseptically to each flask, and the flasks were inoculated as described above.

The content of glucose was determined by the method of Somogyi (1945, 1952) and nitrogen by the Kjeldahl method. Control flasks were analysed immediately after inoculation and each found to contain 241 mg glucose and 0.24 mg nitrogen.

Table 2. *Influence of temperature on the efficiency of nitrogen fixation.*

Strain	Temperature °C	Time of incubation days	Glucose decomposed mg	Nitrogen fixed mg	mg nitrogen fixed pr g. of glucose decomposed
N_1	15	6	212	0.95	4.4
		12	233	1.05	4.5
	25	6	233	0.54	2.3
		12	233	0.58	2.5
N_5	15	6	129	0.50	3.8
		12	233	1.05	4.5
	25	6	227	0.59	2.6
		12	233	0.59	2.5

Table 2 shows a considerably higher amount of nitrogen fixed pr gr of glucose decomposed at 15° than at 25°, whereas the growth rate is somewhat higher at 25° than at 15°.

Influence of pH on nitrogen fixation. Nutrient solutions without carbon source and with 0.5 % K_2HPO_4 were adjusted to various pH values by adding adequate amounts of 1 n HCl, and then sterilized at 120° for 20 minutes; 5 % glucose solution, sterilized by filtration, was added aseptically, and the media were distributed in sterile 100 ml flasks with 25 ml in each. Inoculation as previously described.

Control flasks were analysed immediately after inoculation and found to contain 160 mg glucose and 0.10 mg nitrogen. The culture flasks were analysed after incubation at 25° for 9 days. The results are recorded in Table 3.

The organisms ferment glucose under formation of considerable amounts of acid. At the lower pH-values, where the buffer action of the system is rather slight (pH 4—5), the pH consequently decreases rapidly, and growth is soon inhibited.

At the higher pH-values, where the buffer action is stronger (pH 6—8), the decrease in pH is smaller, and the glucose is exhausted before the limiting pH is reached.

The experiments show that the pH minimum is situated about 3.5—3.6, and no significant difference in growth was established within the range from pH 6 to pH 8.

Apparently the reaction has no appreciable influence on the efficiency of nitrogen fixation.

Influence of oxygen supply on nitrogen fixation. The organisms in question are facultative anaerobes, and able to grow and fix elementary nitrogen both under aerobic and anaerobic conditions. It is to be anticipated, however, that the efficiency of nitrogen fixation will be different under the two sets of conditions, because the course of dissimilation presumably will be influenced by oxygen supply.

The results of a few experiments on this question are shown in Table 4.

Table 3. *Influence of pH on nitrogen fixation.*

Strain	Initial pH	Final pH	Glucose decomposed mg	Nitrogen fixed mg	mg nitrogen fixed pr g. of glucose decomposed
N_1	4.10	3.60	10	0.04	—
	5.10	3.75	30	0.13	—
	6.00	5.30	136	0.46	3.4
	7.00	6.00	158	0.46	2.9
	8.00	6.10	158	0.46	2.9
N_5	4.10	3.55	3	0.03	—
	5.10	3.75	106	0.30	2.8
	6.00	4.70	142	0.43	3.0
	7.00	5.55	160	0.43	2.7
	8.00	6.30	158	0.44	2.8

The cultures were prepared as in experiment 2, incubated at room temperature, and grown partly aerobic, and partly anaerobic in a desiccator, where oxygen was absorbed by an alkaline solution of pyrogallol. A methylene blue solution was used as indicator of anaerobiosis (Manual of Methods, III_{43}—4).

Table 4 shows that the influence of oxygen supply on nitrogen fixation is slight or insignificant. In the case of N_1 the nitrogen fixation was slightly higher under anaerobic than under aerobic conditions, and vice versa in the case of N_5. However, the differences are too small to allow general conclusions.

Discussion

Nitrogen fixation by Aerobacter aerogenes has been claimed repeatedly. Löhnis (1905) and Löhnis and Pillai (1907, 1908), working with organisms classified as Bacterium pneumoniae, but presumably identical with or closely related to A. aerogenes, obtained nitrogen yields about 1—2 mg pr g of carbohydrate in the media. However, this gain in nitrogen was rather small compared with the total nitrogen content of the soil-extract media, used in these experiments. The same objection can be raised against the results stated by Fischer (1916, 1918), and Skinner (1928 a, b), and the nitrogen fixing ability of A. aerogenes has been a matter of doubt, because the nitrogen gains were considered to lie within the limits of error, until lately Hamilton et al. (1953) by means of the isotope technique obtained a slight but consistent fixation of nitrogen by 3 strains of A. aerogenes out of 16 strains investigated.

The nitrogen yields obtained with the two strains, N_1 and N_5, however, are several times higher than those found in any of the earlier investigations. Under optimum conditions these strains were able to fix 4—5 mg nitrogen pr g of carbohydrate decomposed, corresponding to an increase in the total

Table 4. *Influence of oxygen supply on nitrogen fixation.*

Oxygen supply	Time of incubation days	Strain	Initial glucose content mg	Glucose decomposed mg	Nitrogen fixed mg	mg nitrogen fixed pr g. of glucose decomposed
anaerobic	7	N_1	242	238	0.89	3.7
—	—	N_5	—	235	0.59	2.5
anaerobic	14	N_1	205	204	0.86	4.2
—	—	N_5	—	203	0.60	3.0
aerobic	14	N_1	205	199	0.76	3.8
—	—	N_5	—	194	0.69	3.6

nitrogen content of 20 ml nutrient solution from 0.2 to 1.2 mg nitrogen, a nitrogen gain far beyond the limits of error.

Only a small minority of Aerobacter strains, however, are able to fix elementary nitrogen in appreciable quantities, as shown by Skinner (1928 a, b), who found nitrogen fixing ability in only 3 out of 23 strains investigated, and by Hamilton et al. (1953), and no doubt this is the explanation of many negative results.

A third strain, N_3, was isolated together with the strains, N_1 and N_5, and very similar to these (indol-negative), but no nitrogen fixation could be detected in this strain in spite of repeated attemps. Apparently there is no correlation between nitrogen fixing ability and other characteristics, but the closer relations between nitrogen fixing and non nitrogen fixing strains of A. aerogenes as well as the distribution and frequency of the former deserve further studies.

A point of special interest in the physiology of these organisms is the influence of temperature on the nitrogen fixing ability. The experiments show a considerably greater amount of nitrogen fixed at lower ($15°$—$18°$) than at higher temperatures ($25°$—$30°$) in proportion to the amount of nutrients consumed, although the growth rate is distinctly higher at the higher temperature. No satisfactory explanation of this phenomenon can be given for the time being. Apparently the carbohydrates are utilized more economically at lower than at higher temperatures, resulting in synthesis of more cell substance at the lower temperature. Perhaps also a certain accumulation of nitrogen is taking place, but this question will be made the object of further investigations.

Summary

The nitrogen fixing ability of two strains of Aerobacter aerogenes, isolated from two water samples, was studied.

It seems as if some growth factor, present in yeast extract and agar, is

necessary for fixation of molecular nitrogen, whereas the organisms grow well in a fully synthetic medium, when combined nitrogen is present.

The efficiency of nitrogen fixation depends considerably on temperature. The maximum yield was obtained at $15°$—$18°$ and amounted to 4—5 mg nitrogen pr g of carbohydrate decomposed.

Oxygen supply had no appreciable influence on growth and nitrogen fixation, and no significant difference in nitrogen fixation could be established within the range from pH 6 to pH 8. The minimum pH was found about 3.5—3.6.

A simultaneously isolated strain showed no nitrogen fixation, though in other characters it was identical with these two strains.

References

Bergey's Manual of Determinative Bacteriology, 6. ed. Baltimore, 1948.

Duguid, J. P.: The influence of cultural conditions on the morphology of Bacterium aerogenes with reference to nuclear bodies and capsule size. — J. Path. Bact. 60: 265, 1948.

Fischer, H.: Über qualitative und quantitative Leistungen stickstoffsammelnder Bakterien in Wasser und im Boden unter Wasserbedeckung. — Centralbl. Bakt. II Abt. 46: 304, 1916.

— Das Problem der Stickstoffbindung (Festlegung des Luftstickstoffs) bei niederen Pflanzen. — Ber. Deut. Bot. Gesellsch. 35: 423, 1918.

Hamilton, P. B., Magee, W. E. and Mortenson, L. E.: Nitrogen fixation by Aerobacter aerogenes and cell-free extracts of the Azotobacter vinelandii. — Bact. Proc. 1953: 82.

Jensen, V.: The Azotobacter flora of some Danish watercourses. — Bot. Tidsskr. 52: 143, 1955.

Löhnis, F.: Beitrage zur Kenntnis der Stickstoffbakterien. — Centralbl. Bakt. II Abt. 14: 582, 1905.

— und Pillai, N. K.: Ueber stickstofffixierende Bakterien II. — Ibid. 19: 87, 1907.

— — Ueber stickstofffixierende Bakterien III. — Ibid. 20: 781, 1908.

Manual of Methods for Pure Culture Study of Bacteria, ed. by Soc. Amer. Bact. Geneva, N. Y. 1946.

Parnas, J. K.: Über die Ausführing der Stickstoffbestimmung nach Kjeldahl in der Modifikation von Parnas und Wagner. — Zeitschr. anal. Chemie, 114: 261, 1938.

Parr, L. W.: Coliform bacteria. — Bact. Rev. 3: 1, 1939.

Skinner, C. E.: The fixation of nitrogen by Bacterium aerogenes and related species. — J. Bact. 15: 9, 1928 a.

— The fixation of nitrogen by Bacterium aerogenes and related species. — Soil. Sci. 25: 195, 1928 b.

Somogyi, M.: A new reagent for the determination of sugars. — J. Biol. Chem. 160: 61, 1945.

— Notes on sugar determination. — J. Biol. Chem. 195: 19, 1952.

On the Assimilation of Phosphorus in Chlorella pyrenoidosa

By

A. A. AL KHOLY

Red Sea Marine Biological Station, Cairo University
(Received December 1, 1955)

Ketchum (1939) showed how the amounts of phosphorus and nitrogen vary in cells of the plankton algae Nitzschia closterium and Chlorella pyrenoidosa. If these algae were growing in a medium deficient in phosphorus, cells were produced showing a P-content one third of the normal found by him. This phosphorus deficiency could be recovered if phosphate was added to the medium. The absorption of P took place both in light and in the dark. These observations were verified by Mackereth (1953), who used cells of the diatom Asterionella formosa. This author obtained a minimum amount of 0.06×10^{-6} µg P per cell.

In the present article an experimental series with Chlorella pyrenoidosa is published. Some few similar series were made. During these experiments cells deficient in phosphorus were produced and the absolutely possible minimum value of P per cell was ascertained. The absorption of P by deficient cells in light was followed at the same time. The following three P-components were determined: dissolved inorganic phosphate, dissolved organic P, and particulate P. In the present investigation particulate P was identical with the P found in the algae. The sum of these three components gives the amount of total P. The primary purpose of the present investigation was at the same time to follow the uptake of phosphate and the growth of the algae after the addition of phosphate to a P-deficient culture of Chlorella.

It must be considered very important ecologically that plankton algae are able to store phosphorus in their cells. Such species are able to continue growing for some time after complete depletion of the element in the outside medium. It was shown by Mackereth that the cells of Asterionella formosa

have a marked ability to accumulate phosphorus even from very low external concentrations. In the lake Windermere the few cells present can maintain a reserve of phosphorus during the winter which later is used for growth. Later in the year the amount of phosphorus per cell consequently decreases.

Methods and Experiment

Warburg's culture medium was used (5 g $MgSO_4$, 7 H_2O, 2.5 g KH_2PO_4, 2 g NaCl, 2 g KNO_3, 2 g $Ca(NO_3)_2$, 4 H_2O. 6 mg $FeSO_4$, 0.06 mg $ZnSO_4$, 0.06 mg $MnSO_4$ per 1000 ml glass distilled water). The amount of phosphate, which is so high in this medium, was changed to 1000—1270 µg P/L.

Precautions for bacterial contaminations were taken, but it is not certain that the culture was kept totally free of bacteria. A two litre conical flask was used with a cotton plug, through which passes a syphon for sampling and stirring with air containing 5 per cent CO_2. The culture was illuminated continously from beneath with four 20 watt fluorescent lamps. The inoculum for the experiment was taken from a culture deficient in phosphorus.

Counting of the cell number was done by using a blood counting slide. Filtration was carried out under pressure through a collodion membrane filter.

The extinction of the culture in per cent was determined every day by means of a portable E.E.L. colorimeter, using a red filter with a mean wavelength of transmission at 660 mµ.

Inorganic phosphate was determined by using the method of Dénige modified by Atkins (1923). Organic phosphorus, dissolved or particulate, was determined by the method of Harvey (1948). 67 ml of the culture was used, filtered (for dissolved P) or not filtered (for particulate+dissolved P). One ml H_2SO_4 (c. 1 : 1) was added and the samples were autoclaved for 6 hours at 140° C. This time is absolutely sufficient for a complete hydrolysis. Two ml ammonium molybdate (6.6 g ammonium molybdate in 400 ml distilled water+6 ml conc. H_2SO_4) and one drop of stannous chloride (freshly prepared) were added. The stannous chloride was made in the following way: 50 ml concentrated hydrocloric acid containing 2 g metallic tin were completed to 2 ml with distilled water. It was then boiled under reflex condenser till all the metal tin dissolved. The process takes a whole day. The reagent thus obtained was kept under paraffin oil. The blue colour obtained by the addition of the reagents for phosphate was measured by the E.E.L. colorimeter, using the above-mentioned red filter.

When the culture was very dense it was found necessary after autoclaving to filter the previously non-filtered samples. The decomposed organic matter
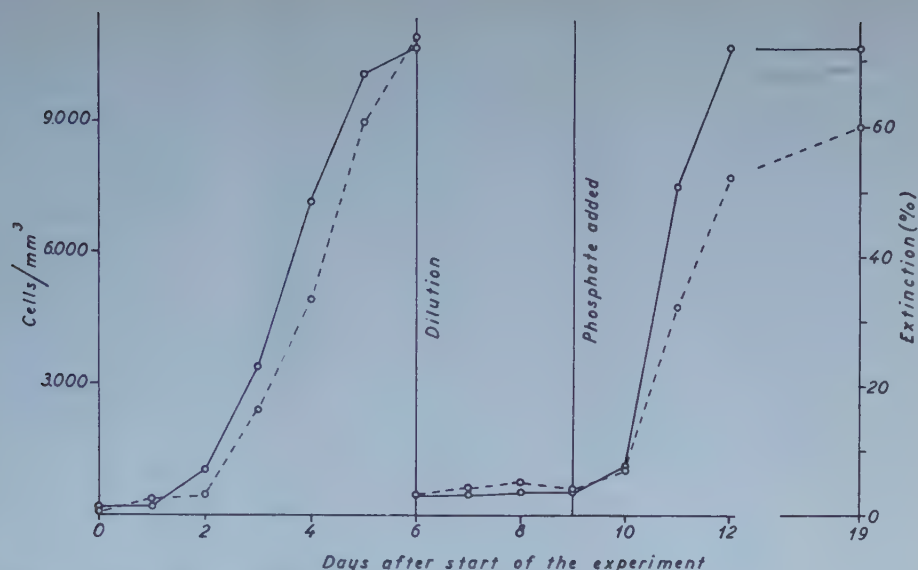
Figure 1. *Growth of Chlorella in the three phases of the experiment.* The solid lines show the number of cells on the different days of the experiment. The dashed lines represent the extinction of the culture.

made the determination of phosphate impossible if no filtration was made. A filter not giving off any phosphate was used. Calibration standard curves were made for phosphate determined directly and after autoclaving

The experiment was divided into three parts. (1) During the first part the culture grew normally for seven days after which the minimum phosphorus content in the cells was reached. (2) After growth had stopped at this limit, the culture was diluted with the same medium, however, containing no phosphate. This dilution was made in order to investigate if growth really stopped due to deficiency in P and not because light had become the limiting factor. The culture was rather dense before dilution. (3) Four days after the dilution phosphate was added. The analysis were continued for four days, after which the minimum value of phosphorus per cell was reached once more and growth stopped. The cell concentration was examined a week later.

The figures presented in the following curves are all based on duplicates. The range of variation was ± 10 μg P/L for dissolved organic and particulate P. The difference between the duplicates of inorganic phosphate were insignificant. The range of variations in total P from day to day was found to be about ± 5 per cent.

Growth, Figure 1. The medium was inoculated with 165 cells/mm³. There was no growth according to the cell counts during the first day. We can thus
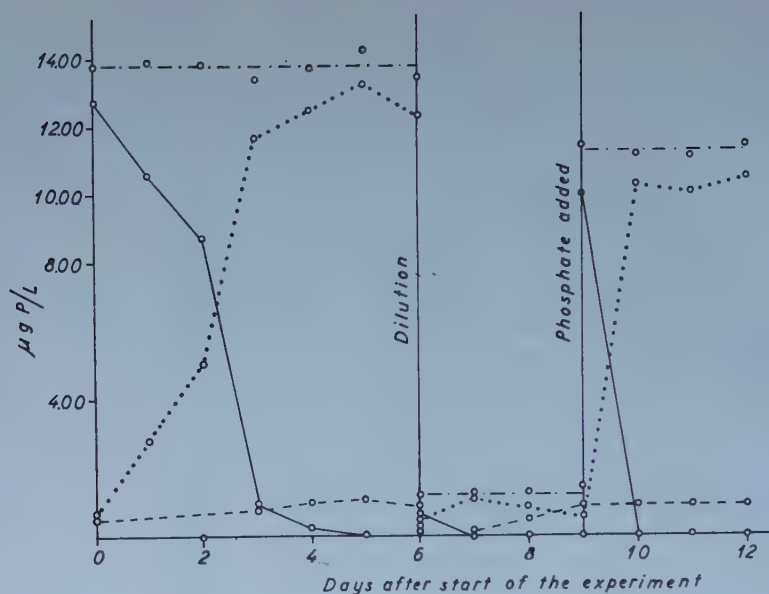
Figure 2. *Concentration of the different P-fractions in the three phases of the experiment.*
The solid lines show the concentration of inorganic phosphate on the different days of the
experiment, the dotted lines represent the particulate P, the dashed lines the dissolved
organic P, and the dash-and-dot lines the total P.

conclude that the cells pass about a day in restoring deficiency in phosphorus
without division. The maximum rate of cell divisions in the species used
under the present conditions is nearly three times per 24 hours. After the
cultures was diluted no real growth took place. The slight increase in cell
number was due to the 60 μg P/L which was introduced as a result of conta-
mination. This indicates that under the present conditions growth was limited
due to shortage of phosphorus in the cells and not due to the light. After addi-
tion of 1000 μg P/L and a lag time growth started again. Three days later the
growth, however, had stopped entirely. A concentration of 10,800 cells/mm³
was found.

Inorganic Phosphorus, Figure 2. The culture medium was at first supplied
with 1271 μg P/L. This amount was consumed in about five days, resulting in
the production of 7035 cells/mm³, but growth did not stop and still 3500
cells/mm³ were produced in the absence of phosphate. The growth that fol-
lowed the disappearance of phosphate from the medium of course took place
at the expense of the phosphate accumulated before in the cells. The 60 μg
P/L appearing immediately after dilution on the 7th day were consumed rapidly
and appeared as particulate P in the determination made the next day. The 1000
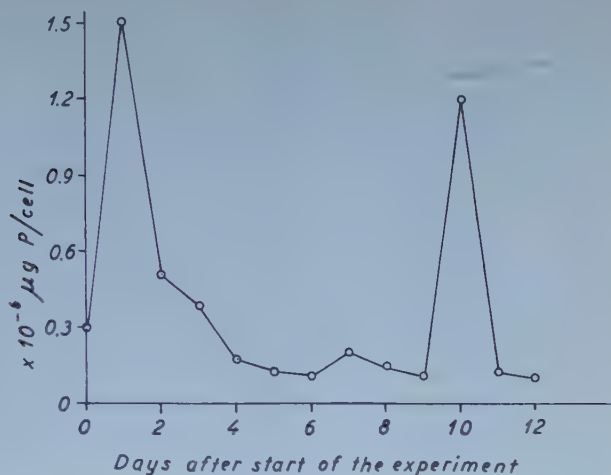
Figure 3. *Variation in the amount of Phosphorus per cell of Chlorella pyrenoidosa during the experiment.*

μg P/L added on the 10th day were consumed during a single day by the cells deficient in phosphorus. On the 11th day an intense growth started again. We can thus conclude that growth proceeds after the complete depletion of phosphate from the outside medium but of course only to a certain limit.

Dissolved Organic Phosphorus, Figure 2. The difference in phosphorus content between the autoclaved filtered and the non-autoclaved filtered sample examined directly for inorganic phosphate gives the value for dissolved organic P. This method was used successfully by Postma (1954). The value 49 μg P/L at the start of the experiment increases slowly but steadily (if we exclude the possibly wrong determination on the third day) until the maximum cell number is obtained. This increase is clearer in the second part of the experiment, after dilution. The concentration of dissolved organic P was constant through the third part of the experiment. It may be suggested that the origin of the dissolved organic phosphate, the nature of which is not known, is either the dead or the living cells. In the latter case, which is rather improbable, it may appear as an excretory product. The rather rapid increase in the second part of the experiment is probably due to an increased death of cells, as the deficiency in phosphorus had now been found for some times. The decomposed cells seem to give off part of their P as dissolved organic P. A similar increase would perhaps have appeared also in the third part of the experiment if the time of the experiment had been somewhat prolonged.

Particulate Phosphorus, Figure 2. The difference in the amount of phosphorus in the autoclaved not filtered and the autoclaved filtered samples gives the amount of particulate phosphorus. The amount of inorganic phosphate that disappears from the outside medium is converted to particulate P. Once

the inorganic phosphorus has disappeared from the medium, the concentration of particulate P is nearly constant. The concentration of total P was constant during the whole experiment.

The Content of Phosphorus in the Cell, Figure 3. As mentioned above the cells used for inoculation were deficient in phosphorus. We might therefore, as shown below, have expected a value of 0.1×10^{-6} µg P/cell at the start of the experiment. This was not the case. The higher value (0.34×10^{-6} µg P/cell) was caused by the fact that the cells rapidly absorbed phosphate during the course of sampling, which took some time. In the third part of the experiment the collection of samples for the determination of the dissolved organic and particulate P was done immediately before the addition of inorganic phosphate. From Figure 3 we can conclude that cells deficient in phosphorus absorb added phosphate very rapidly. In this way they obtain a reserve inside. Growth does not start at once. Immediately after the start of growth the amount of phosphorus per cell decreased although the concentration of inorganic phosphate was still high in the outside medium. As phosphate disappeared from the medium, the value for P per cell decreased until a minimum value was reached. Therefore, as mentioned by Mackereth (1953), analysis of the water can be of little assistance in determining whether or not phosphorus deficiency is limiting growth; on the other hand, analysis of the P content in the cells may give valuable information.

The values for the phosphorus content per cell show two limits, a maximum value which under these conditions was 1.50×10^{-6} µg P/cell and a minimum one which was 0.10×10^{-6} µg P/cell. This minimum value must be considered a very important one as growth cannot proceed when this value is obtained. The maximum value obtained in the third part of the experiment was 1.19×10^{-6} µg P/cell. The amount of phosphate added did not allow the cells to obtain a higher value.

Summary

1: Dissolved inorganic P, dissolved organic P, and particulate P were determined beside the cell number during a growth experiment with Chlorella pyrenoidosa.

2: There is no significant growth of phosphorus deficient cells during the first day after addition of phosphorus to the medium. During that time they restore the deficiency, and phosphorus is further stored inside the cells.

3: The minimum value of phosphorus per cell is 0.10×10^{-6} µg P, once this value is reached, growth stops.

4: If any excretion of organic phosphorus takes place from living cells at all, it is very low in comparison with the total P bound in the cells.

References

Atkins, W. R. G.: Phosphate content of fresh and salt waters in its relationship to the growth of algal plankton. — J. Mar. Biol. Assn. 13: 119. 1923.

Harvey, H. W.: The estimation of phosphate and total phosphorus in sea waters. — J. Mar. Biol. Assn. 27: 337. 1948.

Ketchum, B. H.: The development and restoration of deficiencies in the phosphorus and nitrogen composition of unicellular plants. — J. Cell. and Comp. Physiol. 13: 373. 1939.

Mackereth, F. J.: Phosphorus utilization by Asterionella formosa Hass. — J. Exp. Botany 4: 296. 1953.

Postma, H.: Hydrography of the Dutch Wadden Sea. — Archives Néerlandaises de Zoologie, 10, 4e Livraison. 1954.

Use of ^{14}C-Technique in Measuring Photosynthesis of Phosphorus or Nitrogen Deficient Algae

By

E. Steemann Nielsen and A. A. Al Kholy

Royal Danish School of Pharmacy. Botanical Department, Copenhagen
and Red Sea Marine Biological Station, Cairo University
(Received December 12, 1955)

1. Introduction

Whereas the two techniques used for measuring organic productivity in the sea, the light-and-dark bottle oxygen-method and the ^{14}C-method, in eutrophic waters give corresponding results. this is not the case in the oligotrophic tropical parts of the ocean (Steemann Nielsen 1952, 1954 and Ryther 1954). The oxygen method in these waters gives results according to which these areas would be about the most productive in the world. The values are up to more than 100 times the values obtained by using the ^{14}C-technique. Steemann Nielsen has shown (1954) that the values obtained in tropical oceanic waters by the oxygen method can hardly be supposed to be realistic when the hydrographic conditions of these areas and the scarcity of both phytoplankton and zooplankton are considered (cp. e.g. Hentschel 1933, Jespersen 1935). As most of the light is absorbed by the water and not by the algae the high productivity values must further be considered impossible from an energetic point of view. Steemann Nielsen (1955 a) has finally shown that in oligotrophic waters the difference between the values obtained in light and in the dark by the oxygen method has nothing to do with photosynthesis, but represents a reduction of the bacterial respiration. Immediately sea water is enclosed in small bottles, bacteria start an intensive growth on the walls of the bottles. The production of antibiotics by the algae in light causes a reduction of the bacterial respiration.
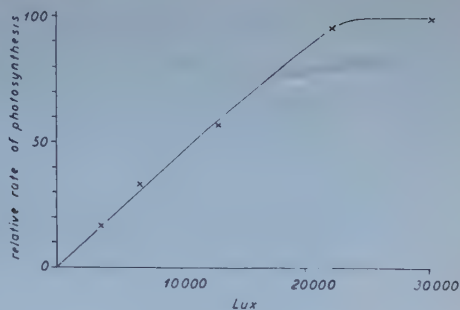
Figure 1. *Relation between light intensity and rate of photosynthesis.* Plankton from oligotrophic, tropical, oceanic water.

Ryther (1954) has advanced the idea that the ¹⁴C-technique only measures net production. He suggests that the disagreement between the two techniques in tropical oceanic water may be due to a combination of the following two suppositions: (1) that algae nearly exclusively respire the newly formed products of their photosynthesis and (2) that the rate of algal respiration is about equal to the photosynthetic rate in these waters. The first suggestion does not agree with experimental evidence. Steemann Nielsen (1955 b) has shown that only about 60 per cent. of the respired carbon is due to newly formed products of the photosynthesis. In the original description of the ¹⁴C-technique it was put at 40 per cent. The ¹⁴C-determinations at least during the "Galathea" Expedition were all made at a light intensity producing a nearly maximum rate of photosynthesis. Ryther's second suggestion would therefore require that the rate of respiration was equal to the maximum rate of photosynthesis. Ryther advances the idea that algae deficient in nutrients behave in this way.

Three light-assimilation curves were made with typically oligotrophic tropical oceanic water during the "Galathea" Expedition. All three series gave the same result. The series presented in Figure 1 is from the western part of the Pacific. The rate of photosynthesis of this plankton under natural light conditions in the sea at the surface was 1.5 mg C per m³ per day, a value only slightly higher than those found in the most oligotrophic areas of the oceans.

The curve presented is a normal one showing the dependence of real assimilation (=gross production) on light intensity. The normal 6 per cent. correction due to intermixing of the photosynthetic and the respiratory processes was made at all light intensities (cp. Steemann Nielsen 1955). If according to Ryther the ¹⁴C-technique only measures net production and the respiratory rate further is practically the same as the maximum photosynthetic rate, the present curve could not be expected. The slope of the curve could not be the same from the lowest light intensities up to about 20,000 lux.

10 · *Physiol. Plant., 9, 1956*

The main object of the present article is to show that the ^{14}C-technique is applicable even if growth is absolutely limited by deficiency either in phosphorus or nitrogen.

2. Measurements of the Rate of Photosynthesis in Chlorella Cultures deficient in Phosphorus or Nitrogen

According to Ryther 1954, ^{14}C experiments in nutrient starved cultures give values which are lower by an order of magnitude compared with the gross production measured by the oxygen technique. This statement has now been reinvestigated by the present writers. We were, however, unable to confirm Ryther's result. Cultures of Chlorella pyrenoidosa either deficient in P or N were used. P-starved cultures were made according to Al Kholy 1956. The P-content per cell was 10^{-7} µg, which seems to be the absolutely lowest content possible in this species. After obtaining this low concentration the culture was kept under natural illumination near a window facing north for 10 days. No P was added to the culture during this time and the concentration of algae remained constant.

N-deficient cultures were produced by adding only 3 mg N/l. KNO_3 to the normal medium at the start of the experiment. 20 mg P as KH_2PO_4 was on the other hand added to the medium. P thus was not limiting. The medium was inoculated with 120 cells per mm³. The cultivation was made for 3 days in continuous light (as used for growing P-starved cells, Al Kholy 1956). Growth had then definitively stopped according to the cell counts (1150 per mm³). The culture was then placed for 6 days more at a window facing north. A mixture of air and 5 per cent. CO_2 was during the whole time blown through the culture.

Experiments with P-deficient cells. In order to get a suitable concentration of algae, 8 ml of the P-deficient culture (11,000 cells per mm³) was mixed with 640 ml of a modified Warburg Medium (without phosphate). Ordinary $KHCO_3$ and 1 ml of a 2 mM $KH^{14}CO_3$ solution was added, giving a titration alkalinity of 2.18 m.equivalent/L and pH 8.1. The algal concentration was thus 88 cells per mm³. Experiments for measuring the rate of photosynthesis and respiration were made with both the oxygen technique and the ^{14}C-technique (only photosynthesis in the case of the latter). Measurements of the dark fixation by the Chlorella suspension were made simultaneously. 15 ml bottles with glass stoppers were used. For the oxygen determination 3 bottles were put in light at 7000 lux. 3 in the dark, and 3 were used as initials. For the ^{14}C-determination 2 bottles were put in the dark and 2 in light. All the bottles with the exception of the initials for the O_2-determina-

tions were placed on a rotating disc in a big water-bath. The whole front wall was glass so that the experimental bottles could be illuminated by twelve 20-watt fluorescent lamps. The temperature was 20° C., the experimental time 4 hours. A semi-micro Winkler titration was used for measuring oxygen. Two determinations of the water from a single bottle could therefore be made. Thus the oxygen determination represents the average of altogether six single determinations. All determinations by the O_2 technique of photosynthesis and respiration are correct up to ± 0.02 mg C/L. The determinations according to the ^{14}C method have a maximum deviation of ± 8 per cent. All values are given in mg C/L per 4 hours. The photosynthetic quotient $\dfrac{O_2}{CO_2}$ is put at 1. This low quotient is used in spite of the fact that 1 is too low under normal conditions in Chlorella. We do not know anything about the quotient under the very special conditions used. The C-values according to the O_2-technique may be assumed to be a little too high.

Rate of photosynthesis,	mg C/L per 4 hours
(a) O_2-technique	0.23
(b) ^{14}C-technique	0.24
Rate of respiration,	mg C/L per 4 hours
O_2-technique	0.09

Dark fixation of $^{14}C = 4$ per cent. of fixation in light.

A parallel series of experiments were made simultaneously in which 2 mg P as KH_2PO_4 were added per L of the experimental water having the same algal concentration as in the series above. This experiment was made in order to investigate whether photosynthesis in Chlorella increases immediately after addition of phosphate to P-deficient cells. The values below as compared with the values above show that this is not the case. These experiments cannot be directly compared with somewhat similar experiments made by Harvey (1933), according to which an addition of phosphate causes an increased rate of photosynthesis as soon as illumination is commenced. After the addition of phosphate in Harvey's experiment, the algae were first kept in the dark overnight. According to Barker (1935). "Harvey's experiments probably give no direct informations concerning the photosynthesis of diatoms".

Rate of photosynthesis,	mg C/L per 4 hours
(a) O_2-technique	0.23
(b) ^{14}C-technique	0.18
Rate of respiration,	mg C/L per 4 hours
O_2-technique	0.08

Dark fixation of $^{14}C = 6$ per cent. of fixation in light.

In addition is further given a series made 2 days before — no P addition. The initials of the oxygen experiment were not titrated due to an accident. It is therefore only possible to state the rate of total photosynthesis. The algal concentration was 110 cells per mm^3 as compared with 88 cells/mm^3 in the two series given just above.

Rate of photosynthesis,	mg C/L per 4 hours
(a) O_2-technique	0.41
(b) ^{14}C-technique	0.34

Dark fixation of $^{14}C = 4$ per cent. of fixation in light.

If the three series are treated jointly, the rate of photosynthesis as measured by the ^{14}C-technique is on an average 88 per cent. of the rate obtained by the O_2-technique. The rate of respiration was 37 per cent. of the rate of photosynthesis. According to Steemann Nielsen (1955) interaction of photosynthetic CO_2 and respiratory CO_2 amounts to about 60 per cent. of the rate of respiration. The measurements of photosynthesis by the ^{14}C-technique were by routine corrected by 6 per cent. according to the supposition that the photosynthetic rate is 10 times the respiratory rate (cp. Steemann Nielsen 1955 b). As however, the rate of photosynthesis in the present experiments was only $\frac{100}{37}$ times higher than the rate of respiration, a correction must instead be made amounting to $\frac{100 \times 60 \times 37}{100 \times 100} = 22$ per cent. If the standard correction — 6 per cent. — already made is substracted, the correction amounts to 16 per cent. As the difference between the values obtained by the two techniques was 12 per cent., an almost perfect agreement is thus found if non-growing Chlorella cells are used which already for 10 days have had the absolute minimum content of P per cell.

The reason why the ^{14}C-technique gave a little higher yield than was to be expected theoretically, is first of all that the rate of dark fixation of ^{14}C — independent of photosynthesis — was relatively high, 5 per cent. as compared with the normal 1 per cent.

Experiments with N-deficient cells. These experiments were made in exactly the same way as the above mentioned experiments with P-deficient cells. Whereas the P-deficient culture was deep green, the N-deficient culture was pale. An algal concentration of 115 cells per mm^3 was used.

(1) No addition to the experimental water of KNO_3

Rate of photosynthesis,	mg C/L per 4 hours
(a) Oxygen-technique	0.13
(b) ^{14}C-technique	0.08

Rate of respiration,   mg C/L per 4 hours

           Oxygen-technique   0.11

         Dark fixation of ^{14}C=37 per cent. of fixation in light.

(2) Addition of 3 mg N/L as KNO_3 to the experimental water

         Rate of photosynthesis,   mg C/L per 4 hours

           (a) Oxygen-technique   0.15

           (b) ^{14}C-technique   0.09

         Rate of respiration,   mg C/L per 4 hours

           Oxygen-technique   0.13

         Dark fixation of ^{14}C=39 per cent. of fixation in light.

In the same way as shown in the experiment in which phosphate was added to P-deficient cells, the addition of nitrate has no or only a slight immediate influence on the metabolism of Chlorella cells which have been N-starving for a long time. It is therefore possible to treat the two experimental series given above jointly. The rate of respiration was 86 per cent. of the photosynthetic rate at the high light intensity of 7,000 lux, which means that the cells apparently during the period covering the last 24 hours under natural light conditions by the window had suffered a loss of organic matter. The rate of respiration per unit number of cells was the same as in the experiments with P-deficient cells and practically the same as in experiments with cells not deficient at all; cp. Winokur 1948. The rate of photosynthesis on the other hand was markedly lower. According to Gregory and Richards (1929) nitrogen deficiency produced slightly decreased respiration in barley leaves, whereas phosphorus deficiency had no effect.

The rate of photosynthesis as measured by the ^{14}C-technique was 61 per cent. of the rate measured by the O_2-technique. Theoretically this value should be $100 - (86 \times 0.6) + 6 = 54$ on the assumption that the photosynthetic quotient is exactly 1 and that the intermixing of photosynthetic and respiratory CO_2 was exactly 60 per cent. of the latter. The value 6 in the equation is due to the fact previously mentioned that a correction for 6 per cent. was already made routinely.

The high rate of dark fixation of ^{14}C is the cause why the ^{14}C-technique gives a higher yield than was to be expected theoretically. Dark fixation is relatively high in algae when the maximum rate of photosynthesis is low.

Even at so extreme conditions that the rate of respiration is almost the same as the rate of photosynthesis, the ^{14}C-technique gives results comparable with the results obtained by using the O_2 method. No indication is found that the rate should be of another order of magnitude.

In the final "Galathea" Report to be published shortly it will be shown that even in the most oligotrophic parts of the oceans the plankton algae

due to the "grazing" by the zooplankton hardly ever reach a state in which the amounts of P and N in the cells approach the absolute minimum values possible in cultures grown in the laboratory. The experiments with oligo-trophic oceanic water shown in Figure 1 thus did not indicate any pro-nounced deficiency in these elements.

3. Ryther's Measurements

As mentioned in Section 2, Ryther in 1954 published the results of some experiments with a marine Chlamydomonas according to which ^{14}C experi-ments gave values which were lower by an order of magnitude compared with the values for the gross production measured by the O_2-technique. It was claimed without giving any details that the experiments were made under conditions of nutrient deficiency. The disagreement with the experi-ments with nutrient deficient algae given in the preceding section might of course be supposed to be due to the fact that another algal species was used. There is not, however, much reason to consider this possibility, as Ryther and Vaccaro (1954) have given an experimental proof of the real cause, which seems to be inadequate experimental technique.

These authors published some experiments according to which measure-ments of photosynthesis by the O_2 and ^{14}C methods gave comparable rates if the experimental period did not exceed 24 hours. ^{14}C experiments con-ducted for period of more than 24 hours, on the other hand gave values lower than the values from O_2 experiments of same duration. They supposed that this was due to the loss of assimilated ^{14}C by respiration. In principle the supposition of an increased loss due to respiration in experiments of long duration is correct, as the ^{14}C method of course will exclusively registre net production after a sufficient space of time when all organic matter found in the algae has the same ^{14}C ratio as CO_2 in the outside medium. This was one of the reasons why the normal experimental time was put at 3—4 hours on the "Galathea"-Expedition.

The explanation mentioned above, is, however, far from being sufficient to explain the results of Ryther and Vaccaro's experiments of longer dura-tion. The ^{14}C values from the experimental series in light given by them in their Figure 2 are especially instructive. In Figure 2 of the present article the results from this series are plotted in a somewhat different way from that in which they were plotted by the writers. The time is still at the abscissa but the C-content of the algae in µg/L is plotted on the ordinate. At the start 40 µg C/L were found in the algae.

The algae increased their C-content during the first 16 hours in light by
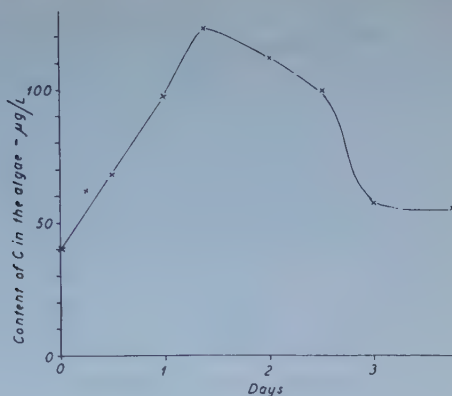
Figure 2. *Ryther and Vaccaro's experiment taking the content of C in the algae into account.*

100 per cent. This is rather normal for plankton algae in good conditions. The C-content increased by 54 per cent. during the next 16 hours, but all increase then stopped completely. Instead a decrease amounting to 9 per cent. was found during the next 16 hour period. From then the decrease accelerated. During the following 24 hours the decrease was 48 per cent. The C-content was now practically the same as at the start of the experimental series.

It may be assumed that the rate of respiration during the first 16 hours was about $^1/_{10}$ of the rate of photosynthesis, i.e. 4 µg/L. As the C-content of the algae during this period on an average amounted to 60 µg/L, the rate of respiration per 24 hours was 10 µg per 100 µg C in the algae. During the third day the average C-content of the algae was 85 µg/L. The decrease in C was 54 µg/L during this period. The minimum rate of respiration — to be found under the absolutely improbable assumption that no photosynthesis at all was going on during this day — was thus 64 µg C per 100 µg C per 24 hours in the algae. Such an enormous rate of respiration must be considered unrealistic. As already shown on p. 149 a possibly arising deficiency in a nutrient does not seem to effect any increase in the rate of respiration.

The results show that something else than respiration and photosynthesis was going on during the latter part of the experiment. This something may most likely be supposed to have been photooxidation which consequently had effected autolysis of part of the algae. According to the experimental conditions these processes may be expected. In the light of our present general knowledge about growing plankton algae and about experiments on photosynthesis and respiration Ryther and Vaccaro's experimental technique can hardly be considered satisfactory. No stirring was effected. In experiments with plankton algae of short duration this may often be adequate, but not if the experiments are going on for long time. Even if sufficient CO_2

on an average is found for photosynthesis in the bottle, local exhaustion of CO_2 will take place due to sedimentation of some of the algae. A chlorophyll sensitized photooxidation is found in algae if they are placed at a high light intensity during CO_2-deficiency (Myers and Burr 1940). Photooxidation may damage some of the cells effecting ultimately a disintegration of the cell content. As neither dissolved organic matter released from such cells nor diminutive solid parts are filtered off by the filters used in the case of the ^{14}C-method, this technique must be considered especially inadequate for measuring photosynthesis under such conditions. The result of Ryther and Vaccaro's experiment just mentioned affords a striking example.

The experiments published by Ryther 1954 were made with the same inadequate technique. Thus there is not much reason to discuss the results which differ so much from those published in the present article.

4. Some Consequenses if Ryther's Suggestions were Correct

The oxygen technique and the ^{14}C-technique disagree by a factor of about 10—200 everywhere in the tropical deep blue parts of the oceans where such comparative investigations have been made. At all the 23 "Galathea" stations with deep blue water, i.e., stations where the depth of the photosynthetic layer exceeded 100 m, the rate of productions as measured with the ^{14}C-technique was without exception low (Steemann Nielsen 1954, Table 1). The rate of photosynthesis was always measured in water samples collected at three different depths of the photosynthetic layer. Not a single measurement with water from any depth showed any resemblance to the results obtained with the O_2 method in this sort of water. During the day algal respiration should therefore according to Ryther's suggestion always be at least 90—99 per cent. of the photosynthetic rate in the bright light at the surface. During 24 hours (a day and a night) the rate of algal respiration in the surface water is twice the rate during the day and must consequently according to Ryther always be at least 180—198 per cent. of the photosynthetic rate there. The rate of photosynthesis in the whole photosynthetic layer is on an average during the day 50 per cent. of the rate at 18.000 lux. which nearly equals to the rate at surface illumination (Steemann Nielsen 1952. p. 132). Computed for the whole photosynthetic layer and for 24 hours the algal respiration should thus always be nearly 4 times the rate of total photosynthesis. It is self-evident that such conditions cannot exist constantly in nature. The dayly loss of organic matter in the oligotrophic Sargasso Sea would correspond to about 10 g C per m^2 surface!

Summary

Experiments with algae either deficient in P or N show that the ^{14}C-technique for measuring photosynthesis (gross production) is also applicable under these conditions. It is demonstrated that the experimental technique was non satisfactory in some similar experiments published by Ryther in 1954 according to which ^{14}C experiments in nutrient starved cultures gave values which were an order of magnitude lower than those obtained by using the O_2-technique.

The suggestion offered by Ryther that (1) the ^{14}C-technique exclusively measures net production and (2) that the rate of net production during the day in the nutrient deficient parts of the oceans is only about 1—10 per cent. of the gross production, which should be measured by the O_2-technique, is shown to conflict with a series of experimental results. Some consequences of Ryther's suggestions are presented.

The cause of the discrepancy between the ^{14}C-technique and the O_2-technique in the tropical, oligotrophic parts of the oceans is found in the fact that the difference in O_2 in the light and in the dark bottles at the end of an experiment has nothing to do with photosynthesis but represents a decrease in bacterial respiration due to antibiotics produced by the algae in light.

References

Al Kholy, A. A.: On the assimilation of phosphorus in Chlorella pyrenoidosa. — Physiol. Plant. 9: 137. 1956.

Barker, H. A.: Photosynthesis in diatoms. — Arch. Mikrobiol. 6: 141. 1935.

Gregory, F. G. and Richards, F. J.: Physiological Studies in plant nutrion. I. — Ann. Botany 43: 119. 1929.

Harvey, H. W.: On the rate of diatom growth. — J. Mar. Biol. Ass. U.K. 19: 253. 1933.

Hentschel, E.: Allgemeine Biologie des Südatlantischen Ozeans. Erste Lieferung. Das Pelagial der obersten Wasserschichten. 1933.

Jespersen, P.: Quantitative investigations on the distribution of macroplankton in different oceanic regions. — Dana Report Nr. 7. 1935.

Myers, J. and Burr, G. O.: Studies on photosynthesis. Some effects of light of high intensity on Chlorella. — Journ. Gen. Physiol. 24: 45. 1940.

Ryther, J. H.: The ratio of photosynthesis to respiration in marine plankton algae and its effect upon the measurement of productivity. — Deep-Sea Res. 2: 134. 1954.

Ryther, J. H. and Vaccaro, R. F.: A comparison of the oxygen and ^{14}C Methods of measuring marine photosynthesis. — Journ. du Conseil Int. l'Expl. Mer. 20: 25. 1954.

Steemann Nielsen, E.: The use of radioactive carbon (C^{14}) for measuring organic production in the sea. — Journ. du Conseil Intern. l'Explor. de la Mer. 18: 117. 1952.

— On organic production in the oceans. — Ibidem 19: 309. 1954.

— The production of antibiotics by plankton algae and its effect upon bacterial activities in the sea. — Pap. in Mar. Biol. and Oceanogr. Suppl. to vol. 3 of Deep-See Res. 1955 a.

— The interaction of photosynthesis and respiration and its importance for the determination of ^{14}C-discrimination in photosynthesis. — Physiol. Plant. 8: 945. 1955 b.

Influence of the Root Pressure on the Transpiration of Wheat Plants

By

HENRY RUFELT

Botanical Laboratory, Lund
(Received January 2, 1956)

Introduction

In i previous paper (2) the author suggested that the intensity of the root pressure has a direct influence upon the transpiration rate of a plant. This assumption was founded partly upon his experimental results and partly upon reports from the literature, that could be interpreted as indicating this connexion. Satoo (15) found that rooted cuttings of Cryptomeria had a higher transpiration than cuttings without roots. Peris (12), Renner (14) and others found a decreased transpiration when the osmotic value of the medium was increased. In the above-mentioned investigation some results indicated a connexion between the root pressure and the transpiration. Firstly it was shown that when a leaf was excised from the plant, with the place of cutting immersed in water, a sudden drop in the transpiration from the leaf was obtained. In order to explain this result it was suggested that the root pressure continuously facilitates the water transport upwards in the plant. Therefore an elimination of this pressure can be recorded as at least a temporary decrease in the transpiration. Secondly when a plant was watered during the experiment a decreased transpiration could be recorded. It could be shown that this result was not depending on the decrease of the temperature and therefore it is most satisfactorily explained as a consequence of a decreased root pressure. This decrease is then called forth by a hindering of the ion uptake to the roots, due to a temporary oxygen deficiency that causes a decreased respiration. Another result from the earlier mentioned paper (2) where the magnitude of the root pressure may have an influence

upon the course of the transpiration curve should also be related. The automatic record of the transpiration after the opening of the stomata produced by illumination showed that this was not constant but had a periodical course. The oscillations of the curve grew less and less pronounced and at last they died out. After that the transpiration remained constant. These variations in the transpiration intensity were explained as resulting from stomatal movements due to a discrepancy between the photoactive opening and the hydroactive closure of the stomata. The tendency of hydroactive closure increases during the opening of the stomata as a consequence of a deficiency in the water supply to the guard cells. This explanation is founded on Stålfelt's opinion that the stomatal aperture is mainly affected by a photoactive system and a hydroactive system, both included in the term "hydrophotic" (19). The explanation of the periodical course of the transpiration has been discussed and accepted by Stålfelt (19).

According to Strugger (18) and Hylmö (9) in the mesophyll of the leaf the water is mainly transported in the cell walls and it may be suggested that the deficiency of the water supply to the guard cells is due to the difficulties the plant has to overcome in supplying its leaves with water. If the root pressure has an importance for the translocation of water upwards in the plant it may be assumed that its variations can be recorded in the oscillations of the transpiration curve. This discussion is founded on the assumption that an unbroken water phase is present in the plant and, therefore, a change in the pressure in the root of a plant is immediately transmitted to the top.

The root pressure is regarded as an osmotic phenomenon called forth by the active accumulation of ions in the root. The water uptake resulting from this ion accumulation may be called the active uptake. As is shown by Hylmö (9), we have in addition a passive uptake of water forced by the transpiration. It is suggested that in the root this uptake is principally localized to the cell walls and is, therefore, probably independent of osmotic factors.

The root pressure may be altered in two ways. Firstly by changing the osmotic pressure in the root medium. This has been undertaken by addition of d-mannitol, a substance which has been shown not to affect the cell metabolism and to have no injurious effect upon the roots (6). Secondly the root pressure may be varied by means of the respiration of the roots, which is the energy source of the active ion accumulation. Therefore a respiratory inhibitor was added to the nutrient solution. Sodium-diethyldithiocarbamate (dieca) in the concentration 10^{-3} M was chosen. This concentration is sufficiently high to inhibit the copper enzymes as well as the cytochrome oxidase (11). Experiments showed that further addition of dieca had no cumulative effect and, therefore, it was assumed that the active uptake of salts and water is completely inhibited in this concentration. Neither the addition of

mannitol nor of dieca should affect the passive uptake of water. In the two series of experiments the effects upon the transpiration were studied in especially three respects: the immediate effect of the treatments, the effect of cutting off the leaves under water, and the shape of the transpiration curve after illumination of pretreated plants.

Material and Methods

The experiments were performed with wheat plants (Weibull's Eroica Wheat) which were cultivated in a nutrient solution containing $Ca(NO_3)_2$ 10^{-3} M, KNO_3 10^{-3} M, $MgSO_4$ 5.10^{-3} M, KH_2PO_4 10^{-3} M and Fe citrate 10^{-5} M. The plants were cultivated in glass beakers under constant conditions (21° C, light from 100 W fluorescent lamps) and were about one week old when they were used for the experiments. For each experiment 6—8 plants were employed.

For the transpiration measurements the corona hygrometer was used. This method has been earlier described and discussed (1, 2). Air of constant humidity is passed over the leaves which are enclosed in a cuvette, and after that conducted directly to the corona chamber. Here it passas a streamer corona discharge, which is dependent on the humidity of the air. In this manner the humidity can be measured and the output of water from the leaves can be continuously recorded.

Results

As has already been mentioned the effect of an increased suction force in the medium and the effect of the respiration inhibitor are studied mainly in three respects: firstly the immediate effect upon the transpiration, secondly the changes in the transpiration as a consequence of the excision of the leaves under water, and thirdly the course of the transpiration curve after the stomata have been opened by illumination.

a. *The immediate effect*

In the experiments described in this section the plants have always been illuminated sufficiently long for the earlier mentioned oscillations in the transpiration to disappear before the experiments were started. An automatic record of a typical experiment is shown in figure 1 A where the osmotic value of the root medium is suddenly increased. At the arrows a certain volume of the nutrient solution was removed by means of a pipette and the same volume of a solution of mannitol was immediately added. The volume of the root medium was 250 ml and in most cases 25 ml of the solution was exchanged. The manipulation requires about 10 seconds. All the time air
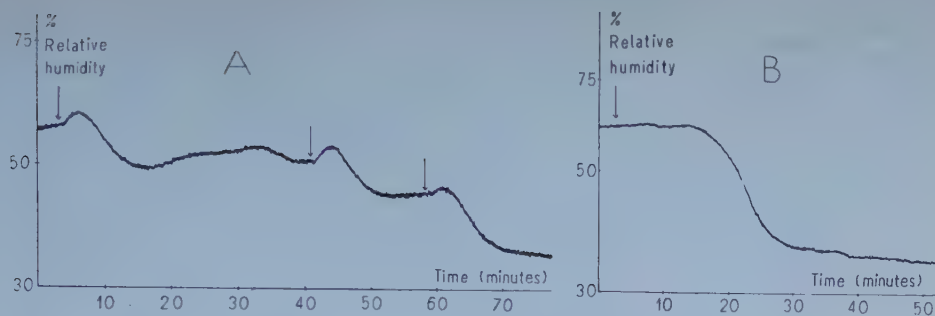
Figure 1. *Automatic records of the transpiration from wheat plants.*
A. Influence of an increased suction force in the root medium. At the arrows the osmotic value of the nutrient solution is increased by addition of D-mannitol. At the first arrow the solution is made 0.1 *M*, at the second 0.2 *M* and at the third 0.4 *M*.
B. Influence of a respiratory inhibitor, sodium-diethyldithiocarbamate. At the arrow the nutrient solution is made 10^{-3} *M* of the inhibitor.

was bubbled through the solution and the mannitol was immediately diluted to a homogenous solution.

It is seen in the transpiration curve that an increase of short duration is immediately obtained, but this is soon changed to a decrease. The decrease sets in after about 5 minutes. After the decrease the transpiration attains a constant value with rather weak oscillations. If the operation is repeated the same phenomena appear again. However, the sudden increase in the transpiration that is obtained immediately after the addition of the mannitol cannot be called forth under all circumstances. Firstly, it could be shown that the peak always appeared if a certain time (15—20 minutes) was allowed to elapse between two additions, but when the addition was repeated at shorter intervals it did not occur. Secondly, it was not recorded when mannitol was added to dieca-inhibited roots. This phenomenon will be discussed later. The magnitude of the later ensuing decrease in the transpiration could be shown to be related to the concentration of the mannitol added (Figure 1 A). Both effects on the transpiration called forth by mannitol could be recorded within the concentration limits of 0.02—0.4 *M* mannitol in the root medium. More diluted or more concentrated solutions have not been tested.

The effect of dieca is illustrated in figure 1 B. Here a decrease in the transpiration sets in after a period of 8—12 minutes. Even in this case the transpiration reached a constant value. Neither in this case nor in that of mannitol was the stomatal transpiration totally inhibited. This could be demonstrated by a darkening of the leaves. Then the transpiration decreased to the value recorded before the illumination.
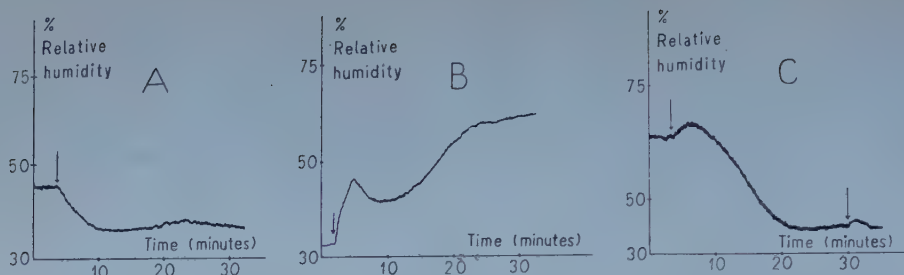
Figure 2. *Automatic records of the transpiration from wheat leaves at the arrow excised from the plant with the place of cutting immersed into water.*

A. Excision from intact plants.
B. Excision from plants with roots inhibited by sodium-diethyldithiocarbamate. Just before the excision the plants are transferred to distilled water.
C. Excision from intact plants but the transpiration is first diminished by adding mannitol to the root medium. Mannitol is added at the first arrow and the excision is undertaken at the second.

b. *The effect of excision of the leaves under water*

Also in these experiments the plants were illuminated until the transpiration became constant before the operations were undertaken. The effect upon the transpiration when intact plants were cut off under water is shown in figure 2 A. A rather great drop is obtained, after which weak oscillations take place. On the contrary an increase is recorded if plants pretreated with dieca are used (figure 2 B) or if mannitol is added to the solution (figure 2 C). In the latter case the peak is obtained only if the osmotic value in the root medium is made rather high. In 0.2 M mannitol it is almost absent and a very weak immediate effect of the excision was recorded. In lower concentrations a decrease is obtained.

After the peak a slow increase in the transpiration was usually recorded. However, the further course of the transpiration is dependent upon the solution taken up by the leaves. If the plants previous to the excision are transferred to distilled water, the transpiration attains a rather high value as is shown by the curve in figure 2 B. On the other hand, if the solution contains salts, the increase is weaker, and if mannitol is present in a high concentration, the increase is very soon changed to a decrease.

c. *The course of the transpiration after illumination*

In all the experiments the light source employed gave a light intensity of about 65,000 Lux. The course of the transpiration after illumination of plants treated in different manners is shown in figure 3. In all cases the time of the
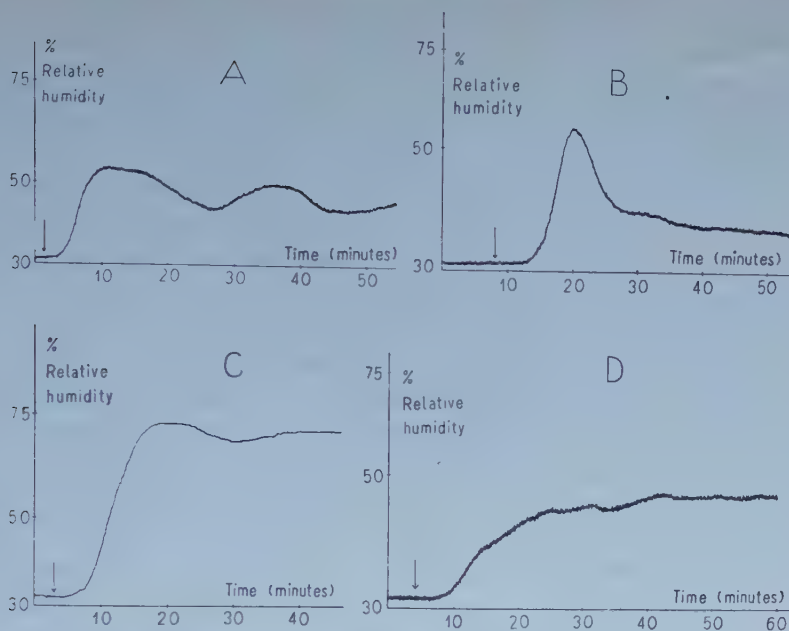
Figure 3. *Automatic records of the transpiration from wheat plants which are illuminated from the moments marked with arrows.*

A. Intact plants.
B. Intact plants with the roots inhibited by sodium-diethyldithiocarbamate.
C. Excised leaves standing in distilled water.
D. Excised leaves standing in a nutrient solution.

onset of illumination is marked by an arrow. Curve A gives the curve of intact plants. Following the initial increase in the transpiration the curve assumes a periodical course. After about two hours it becomes constant which is recorded as a straight line. In curve B the plants are treated with dieca and it is assumed that in this case the root pressure is inhibited. Here the transpiration increases normally as the stomata open, but after 10—15 minutes it drops to a low value and the oscillations are almost absent. The curves C and D deal with records of the transpiration from excised leaves. In C they were placed in distilled water, and here the transpiration attaines rather promptly a constant value without great oscillations. In curve D the leaves have been left in the nutrient solution. The increase after illumination sets in normally, but after about 10 minutes the curve suddenly changes direction. The transpiration continues to increase but rather irregularly, and no peak is observed comparable to those present in the three other curves. After about one hour the transpiration begins to decrease slowly and irregularly.

Discussion

Because it must be presumed that the transpiration rate directly illustrates the movements of the stomata, it can be concluded from the shape of the curve, illustrating the transpiration intensity after illumination, that in normally occurring light intensities the width of the stomata is determined by the water deficit in the leaf as well as by the light intensity. As the stomatal aperture is of great importance for the transpiration intensity it may be concluded that this is strongly affected by the water deficit, which in its turn varies with the water supply to the leaves. Therefore it is only logical to assume that the transpiration rate is directly influenced by the uptake and transport of water, even if it is possible that a high transpiration gives rise to an increased water uptake to the roots. On the other hand, if a high transpiration produces a higher uptake of water, it will also cause an increased transport velocity in the stem, which gives a higher resistence due to friction. However, in order to obtain a clearer understanding of the results presented here it seems to be appropriate to discuss the importance of the water transport upwards in a plant.

As to the driving forces the stream from the roots to the leaves may be divided into two parts. One part is due to the root pressure and the uptake is of osmotic nature. The other part is driven by the transpiration and is not osmotic. The eventually occurring mutual influence of the two parts is then neglected. As to the uptake of water by the guard cells of the stomata, the magnitude of this must be dependent on the supply of water to the walls of the cells surrounding the guard cells. This also imports that it must be dependent on the osmotic value of the solution in their cell walls.

The decrease of the transpiration appearing after the addition of mannitol or dieca must be interpreted as due to a decrease in the water supply to the leaves. The difficulties in the translocation depend on the fact that the root pressure is diminished in the first case and eliminated in the second. This gives rise to an increased water deficit in the leaves, which is followed by a closing of the stomata. That the induction period is longer in the experiments with dieca is quite in accordance with the expectations, because it must be presumed that the decreased difference in the suction forces between the root cells and the medium, which is the ultimate cause to the decreased uptake of water, in these experiments is preceded by a series of reactions. The transient increase in the transpiration caused by the addition of mannitol is more difficult to understand. Of course it could be imagined to be a consequence of the plasmolysis of the root cells. However it is not, which is shown by the fact that it fails to appear when mannitol is added to dieca-inhibited roots. It was shown by experiments that such roots are still plasmo-

lyzable 15 hours after the addition of dieca. Naturally the effect of the plasmolysis upon the transpiration rate must be the same whether the respiration is inhibited or not and it can be stated that the transient increase is not due to the plasmolysis. Some reports in the literature, however, may give a hint as to the nature of the phenomenon. Haines (7) and Humphries (8) have shown that a weak negative pressure in the conducting system of excised shoots of Tilia and Acer gives a temporary increase in the transpiration rate. If the experimental time exceeds $^1/_2$—1 hour the increase is masked by the following decrease. Brewig (5) has found an increased uptake of water through the root caused by an increased transpiration. This phenomenon is interpreted as indicating a change in the permeability for water in the root. Bauer (4) has put the results of Haines and Humphries and those of Brewig on a par with each other and interpreted them as depending on an increased water permeability of the protoplasm caused by an increased suction. These results may be connected with a discovery of Stocker (16, 17) that an increased permeability for water is obtained in the early stages of the drying of cells. In the present results it is not possible to decide whether the liberation of water, which may be the reason for the transient increase in the transpiration, is localized to the roots or to the leaves and it cannot be explained in detail. The quantity of the excess of water, however, can be calculated to about 1 or 2 per cent of the total water content of the plant and, therefore, it has no quantitative influence upon the normal transpiration.

In the second series of experiments, where the leaves are cut off under water, the change in the transpiration must be assumed to be affected by two factors: the root pressure and the filter resistance in the root. These two factors counteract each other and their mutual magnitude will determine the direction of the jump in the transpiration curve. When the root pressure is high enough to overcome the resistance, encountered under the prevailing velocity of water translocation in the plant, the movement upwards in the stem is driven by the root pressure and the transpiration together. In this case the excision at the actual moment will cause a slower water movement and a decreased water supply to the leaves. As a consequence of this the transpiration will decrease. This is illustrated in the experiment with intact plants. (Figure 2 A.) If the root pressure is lower the pressure stream may be eliminated by the filter resistance. The translocation of water in the stem is then driven by the suction of the transpiration only. In this case the excision will faciliate the supply of water to the leaves and the transpiration will increase. This occurs when the root pressure is inhibited with dieca or if the water uptake is greatly retarded by a high osmotic suction force in the root medium (figure 3 B and C).

The water relations and their influence upon the stomatal movements are

11

well illustrated by the course of the transpiration after illumination of the leaves as is recorded in the third series of experiments. In normal cases (figure 3 A) oscillations take place. This is assumed to be a direct consequence of an unsufficient supply of water to the leaves and the guard cells. The deficiency, which sets in after the opening of the stomata, is made up only after a certain time and a reopening is possible. If the supply is strongly impeded the deficiency is filled only very slowly if at all. The consequence of this will be that oscillations do not take place and the transpiration remains at a low level. This is illustrated in the experiment with plants inhibited with dieca (figure 3 B). In the excised plants standing in the nutrient solution (figure 3 D) the water uptake in the beginning is not hindered at all. The transpiration increases normally. After about 10 minutes, however, the increase is suddenly diminished. It may be suggested that here something blocks the water uptake, not the uptake to the leaves but to the guard cells of the stomata. This hindering effect is assumed to be exercised by the increased osmotic value in the solution contained in the cell walls surrounding the stomata. Experiments with a fluorescent dyestuff (the sodium salt of 3-oxypyren-5,8,10-trisulfonic acid) have shown, that this substance is translocated in the conducting elements to the top of the wheat leaves in 2—3 minutes. Of course it is impossible to decide the moment when enough stomata have been reached by salts, in sufficiently high concentration to evoke a closure which can be recorded as a decreased transpiration. A period of ten minutes however, must be regarded as long enough. The interpretation of curve 3 D is supported by the experiments with excised plants standing in distilled water (figure 3 C). Here we get a high degree of opening of the stomata because we have a low osmotic value in the cell walls surrounding the guard cells. Furthermore the hydroactive closure is very weak because the leaves are easily supplied with water. Therefore the oscillations are very low and the transpiration will rapidly assume a constant value.

Summing up it can be stated that the results are in agreement with the opinion that the water uptake is to a certain extent driven by the transpiration. In all the experiments where the root pressure is inhibited the transpiration still takes place. The same is true in the experiments where the root cells are plasmolyzed in a 0.4 M solution of mannitol. The conclusion must be that the water is taken up in some degree nonosmotically and that this portion is transported in parts of the root cells that are not semipermeable. Of course it is most probable that it is localized to the cell walls. The experiments also speak in favour of the opinion that an unbroken water column is present in the plant. Therefore the diminished flow of water into the root is immediately detected in the leaves as a decreased supply, which in turn may be recorded as a decreased transpiration. In this connexion it should

be mentioned that an immediate effect upon the bleeding, called forth by an increased osmotic value in the medium is found by Arisz, Helder, and Van Nie (3). In the present experiments it is indisputable that the root pressure really increases the transpiration and it may be suggested that a very low root pressure has a lowering effect. However, the wheat plants employed are grown under optimal conditions and certainly have rather a high root pressure. Therefore, it is not possible to decide whether the root pressure under normal, less favourable circumstances has a direct influence upon the transpiration rate, and the ecological significance of this factor can only be decided experimentally.

It has earlier been pointed out by the author (2) that the method of measuring the transpiration by weighing excised leaves is not reliable under all circumstances. It could be shown that the sudden increase in the transpiration, which is obtained from a leaf immediately after its excision from the plant may have considerable influence upon the quantity of water given off. This increase was interpreted by Iwanoff (10) as due to the release of a stress in the conducting elements. The magnitude of the increase depends upon the length of the piece of the petiole left on the lamina. Rawitscher (13) has lately published determinations of the transpiration from leaves at the very moment of excision, without finding an Iwanoff chock. However, the details of the technique are not given in his paper and therefore his results cannot at present be compared with ours. The fact that the root pressure under circumstances may have an influence upon the transpiration rate also limits the possibilities of taking the loss of water from excised leaves as a measure of the transpiration, and it must be emphasized that this method should be used only with the greatest care.

Summary

The aim of the investigation was to solve the problem of whether the root pressure has a direct influence upon the transpiration rate of the plants. Wheat roots grown in a nutrient solution under constant conditions were used for the experiments. The root pressure was inhibited by treating the roots with a respiration inhibitor, sodium-diethyldithiocarbamate, and counteracted by increasing the osmotic value of the root medium by addition of mannitol. The effects of the treatments were studied in three respects:

a. The immediate effect upon the transpiration. The transpiration was decreased as a consequence of the treatments.

b. The effect upon the transpiration of excision of the leaves with the place of cutting immersed in water. The normal consequence of the excision

of intact plants is a decreased transpiration. When the root pressure is inhibited or sufficiently diminished an increased transpiration was obtained.

c. Automatic records of the transpiration after illumination of the leaves. These were carried out with plants treated in the two above-mentioned manners. The results illustrate very well the importance of the root pressure.

The results are discussed and the conclusion is drawn that for the wheat plants used in the experiments the root pressure clearly increases the transpiration.

References

1. Andersson, N. E. & Hertz, C. H.: Positive Spitzenentladung als Hygrometer geringer Trägheit. — Zeitschr. f. angew. Physik 7: 361. 1955.
2. — — & Rufelt, H.: A New Fast Recording Hygrometer for Plant Transpiration Measurements. — Physiol. Plant. 7: 753. 1954.
3. Arisz, W. H., Helder, R. J. & Van Nie, R.: Analysis of the Exudation Process in Tomato Plants. — Journ. Exp. Bot. 2: 257. 1951.
4. Bauer, L.: Zur Frage der Stoffbewegungen in der Pflanze mit besondere Berücksichtigung der Wanderung von Fluorochromen. — Planta 42: 367. 1953.
5. Brewig, A.: Beobachtungen über den Einfluss der Sprossaugung auf die Stoffdurchlässigkeit der Wurzeln. — Ber. deutsch. bot. Ges. 54: 80. 1936.
6. Burström, H.: Studies on Growth and Metabolism of Roots. IX. Cell Elongation and Water Absorption. — Physiol. Plant. 6: 262. 1953.
7. Haines, F. M.: Transpiration and Pressure Deficit. IV. — Ann. Bot. 50: 283. 1936.
8. Humphries, E. C.: The Effects on Rates of Transpiration and Absorption of Small Variations in the Pressure in the Xylem Tracts. — Ann. Bot. N.S. 2: 665. 1938.
9. Hylmö, B.: Transpiration and Ion Absorption. — Physiol. Plant. 6: 333. 1953.
10. Iwanoff, L.: Zur Methodik der Transpirationsbestimmung am Standort. — Ber. Deutsch. Bot. Ges. 46: 306. 1928.
11. James, W. O.: The Use of Respiratory Inhibitors. — Ann. Rev. of Plant. Physiol. 4: 59. 1953.
12. Peris, K.: Zur potometrischen Bestimmung der Permeabilität der Wurzeln intakter Pflanzen. — Protoplasma 26: 587. 1936.
13. Rawitscher, F.: Beobachtungen zur Methodik der Transpirationsmessungen bei Pflanzen. — Ber. Deutsch. Bot. Ges. 68: 287. 1955.
14. Renner, O.: Versuche zur Bestimmung der Filtrationswiderstandes der Wurzeln. — Jahrb. wiss. Bot. 70: 805. 1929.
15. Satoo, T.: Water Intake by Cuttings of Sugi. — Journ. Jap. Forest. Soc. 32: 287. 1950. Summary in Biol. Abs. Sekt. D. 1951. No. 21544.
16. Stocker, O.: Probleme der pflanzlichen Dürreresistenz. — Naturwissenschaften 34: 362. 1947.
17. — Beiträge zur einer Theorie der Dürreresistenz. — Planta 35: 445. 1948.
18. Strugger, S.: Der aufsteigende Saftstrom in der Pflanze. — Naturwissenschaften 31: 181. 1943.
19. Stålfelt, M. G.: The Stomata as a Hydrophotic Regulator of the Water Deficit of the Plant. — Physiol. Plant. 8: 572. 1955.

The Assimilation of Ammonia and Nitrate by Nitrogen-Starved Cells of Chlorella vulgaris. IV. The Dark Fixation of Carbon Dioxide

By

P. J. SYRETT

Botany Department, University College, London
(Received September 29, 1955)

Introduction

Nitrogen-starved cells of *Chlorella vulgaris* rapidly assimilate added ammonium and nitrate-N to organic nitrogen compounds; the necessary carbon is supplied by intracellular polysaccharide. An increased respiration rate accompanies the nitrogen assimilation and, when ammonium-N is assimilated, one carbon atom is released as carbon dioxide for every three atoms which are converted to organic nitrogen compounds (Syrett, 1956).

At the present time, it appears that carbohydrates are converted to amino-acids via the organic acids of the Krebs tricarboxylic acid cycle (see Figure 2). the chief organic acids used for amino-acid synthesis being pyruvic. oxalacetic and α-ketoglutaric acids, especially the last. A consideration of the cycle shows that the α-ketoglutaric acid removed by synthetic reactions is replaced, via citric acid. by the condensation of acetate and oxalacetate and. as far as is known, oxalacetate can only be formed from carbohydrate via pyruvic acid which must be carboxylated. This argument suggests that the assimilation of ammonium-N should be accompanied by the fixation of a considerable amount of carbon dioxide which is necessary for the synthesis of the organic acid precursors of amino-acids. Ochoa (1951) and Fowler and Werkman (1952) have emphasised the importance of carboxylation reactions in such synthesis.

By allowing cells to assimilate ammonium-N while in an atmosphere

enriched with $^{14}CO_2$ the dark fixation of carbon dioxide has been measured in the experiments described here. The volume of carbon dioxide fixed was about one third of that produced by respiration.

Material and methods

Chlorella vulgaris (Pearsall's strain) was grown and starved of nitrogen as described in the preceding paper. For the experiments the cells were washed and suspended in 0.067 M phosphate with 0.0017 M magnesium sulphate, pH 6.1. The cell suspension contained about 8.0 mg. dry wt. cells per ml. All experiments were carried out in darkness and at 25° C.

Double-armed Warburg flasks were used for the experiments. 2.0 ml. cell suspension was placed in each flask. One side-arm contained 0.2 ml. sodium carbonate solution containing about 2.5 μcuries of radioactive carbon and 360 μl. of carbon dioxide; after the flasks had been attached to their manometers and placed in darkness, carbon dioxide was liberated from the sodium carbonate by the addition of acid through the side-arm gas-vent stopper. The second side-arm contained either ammonium sulphate solution (37.5 μ-atoms N), potassium nitrate (18.8 μ-atoms N) or nitrogen-free buffer. These solutions were added to the cells one hour after the liberation of carbon dioxide into the flasks.

At intervals after the addition of the substrates flasks were removed and 2.0 ml. of the cell suspension in them pipetted into centrifuge tubes. The cells were centrifuged, washed once and then heated for 3 minutes at 100° C. About 5 minutes elapsed between sampling the flasks and killing the cells. The killed cells were resuspended in 0.9 ml. 60 % ethanol and extracted in this at room temperature overnight.

Measurement of gas exchange

Oxygen uptake and carbon dioxide production during the experimental period were estimated from Warburg manometers containing the same quantities of cells and substrates as those used for the sampling experiments. These flasks, however, unlike the experimental ones with $^{14}CO_2$, did not contain 2 % carbon dioxide at the beginning of the experimental period. The method of calculating carbon dioxide production is described in the preceding paper.

Determination of radiocarbon fixed by cells

0.025 ml. samples of the suspension of cells in 60 % ethanol were dried on lens tissue discs on aluminium trays (Calvin, Heidelberger, Reid, Tolbeck and Yankwich, 1949). Preliminary determinations showed that reproducibility by this method was good. A proportional gas (methane) flow counter was used for counting.

After counting the whole suspension the cells were removed by centrifuging and samples of the extract were counted.

The total amount of radiocarbon liberated in the flasks was calculated by counting the original sodium carbonate solution, after dilution, on tissue discs impregnated with barium hydroxide.
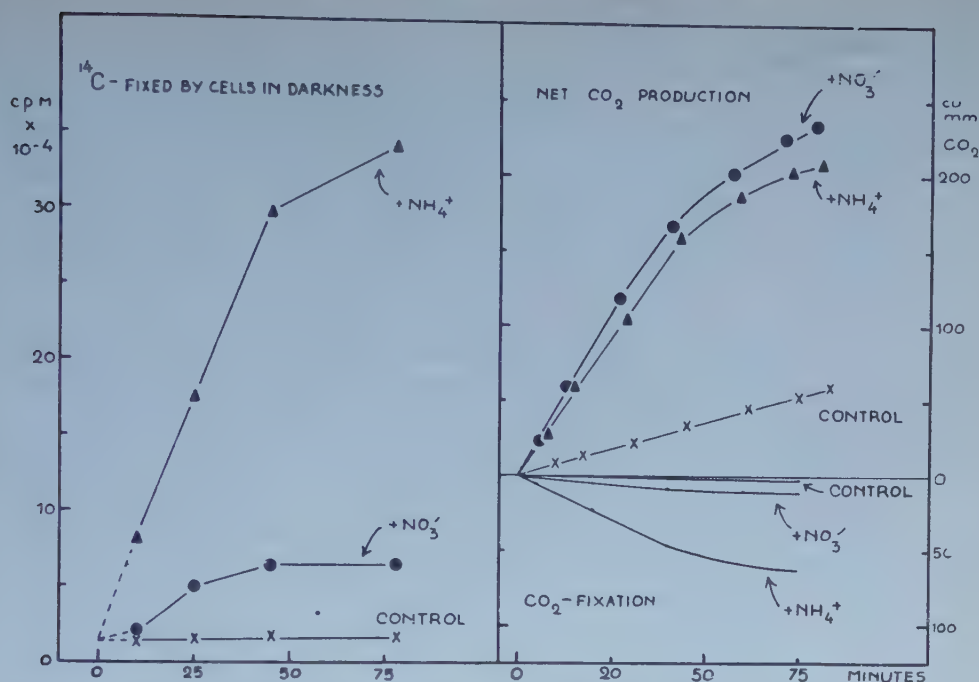
Figure 1. (a) ^{14}C fixed by nitrogen-starved cells of Chlorella while assimilating ammonium or nitrate-N. (b) Upper part — carbon dioxide production of cells measured by manometry; lower part — calculated carbon dioxide fixation. The nitrogen source was added at zero time. All results are given for 2.0 ml. cell suspension which contained 16.2 mg. dry wt. cells. pH 6.06.

The initial volume of carbon dioxide in the flasks was calculated from the volume of carbon dioxide liberated from the sodium carbonate when acid was added to it. To this volume were added the volume of carbon dioxide dissolved in the cell suspension (determined by adding acid to samples) and the volume of carbon dioxide calculated to have been produced by respiration during the interval between placing the cells in the flasks and the addition of the substrates. The total volume of carbon dioxide was about 400 µl. From this volume and the radioactivity of the sodium carbonate solution the initial specific activity of the carbon dioxide in the flasks was calculated.

Results

Three experiments were carried out; the results of a typical one are shown in Figure 1. Little $^{14}CO_2$ was fixed by the control cells during the experimental period, more was fixed by the cells which assimilated nitrate and much more by the ammonium cells. The amount of ^{14}C fixed cannot be directly con-

Table 1. *Distribution of* ^{14}C *fixed by Chlorella during nitrogen assimilation.* ^{14}C fixed is given as counts per min. per flask ($\times 10^{-4}$). Each flask contained 16.2 mg. dry wt. cells.

Time from addition of N-source (mins.)	Control		NO_3'-N added		NH_4^+-N added	
	^{14}C Fixed	% Soluble	^{14}C Fixed	% Soluble	^{14}C Fixed	% Soluble
8	1.26	83.8	1.96	81.6	8.43	82.5
22	1.46	83.4	5.02	75.1	17.88	72.0
43	1.70	81.2	6.35	64.3	29.97	64.0
77	1.64	80.0	6.70	58.0	34.43	56.1

verted into a volume of carbon dioxide since allowance must be made for the continuous dilution of the $^{14}CO_2$ in the flasks by the $^{12}CO_2$ produced by respiration. However, the change in specific activity by dilution can be calculated and hence the volume of carbon dioxide fixed can also be calculated. This is shown in Figure 1 b — the upper part of the figure shows the net carbon dioxide production and the lower part, the carbon dioxide fixation. The *total* volume of carbon dioxide produced by the cells must be equal to the sum of the net production and the volume fixed.

Much of the ^{14}C fixed was in soluble compounds; the proportion converted to insoluble compounds increased with the length of the experimental period (Table 1).

Radioautographs of two-dimensional chromatograms of the extracts from the ammonium cells showed that most of the soluble ^{14}C was in glutamic acid, aspartic acid, glutamine and basic amino-acids.

Discussion

Figure 1 shows clearly that cells assimilating ammonium-N rapidly fix carbon dioxide in darkness; the rate of fixation is about 15 per cent of the rate of photosynthesis of similar nitrogen-starved cells. Cells assimilating nitrate-N fix carbon dioxide more slowly — at one-fifth of the rate of the ammonium cells. It is interesting that the ammonium cells also synthesize organic nitrogen compounds 4—5 times more quickly than nitrate cells (Syrett, 1956).

The rate of carbon dioxide fixation by the ammonium cells is about 33 per cent of the measured rate of carbon dioxide production; this suggests that the gross rate of decarboxylation is 33 per cent greater than that measured by manometry. However, this is only an approximate estimate for several reasons. Firstly, the rate of carbon dioxide production was calculated from flasks with no added carbon dioxide whereas 2 % carbon dioxide was released in the experimental flasks with the $^{14}CO_2$. There is some indication

that the rate of carbon dioxide production is slightly higher in the presence of 2 % carbon dioxide. Secondly, it is possible that the non-radioactive carbon dioxide produced by the cells is closer to the sites of carbon dioxide fixation so that some of it is fixed before it equilibrates with the total carbon dioxide in the flasks. If so, the volume of carbon dioxide fixation calculated from the amount of radiocarbon in the cells will be smaller than the true amount. Thirdly, it has been assumed that all the carbon dioxide produced by the cells is non-radioactive; this assumption will not be true in the later stages of the experiment. Lastly, there is the possibility of rapid exchange reactions in the ammonium cells. However, there is no reason for supposing that the errors from these sources are large.

The net rate of carbon dioxide production of the nitrate cells is always slightly higher than that of the ammonium cells. However, when allowance is made for the greater rate of dark carbon dioxide fixation by the ammonium cells the rate of decarboxylation of the ammonium cells is the greater.

As was predicted from a consideration of the role of the tricarboxylic acid cycle in synthesis, much carbon dioxide is fixed when ammonium-N is assimilated. This does not, of course, prove that the cycle is so involved. However, if one assumes that it is involved, an interesting difference between the decarboxylation reactions of the nitrate and ammonium cells appears. The ammonium cells synthesize considerable amounts of glutamic acid, glutamine and basic amino-acids, especially arginine (Syrett and Fowden, 1952) which is presumably synthesized from α-ketoglutarate via glutamic acid (Greenberg, 1954). Such synthesis will rapidly remove α-ketoglutarate from the cycle, and consequently, the carbon dioxide produced by the decarboxylation of isocitrate to α-ketoglutarate must be an important part of the total carbon dioxide production of the ammonium cells. In the nitrate cells, where the rate of synthesis is slower, less α-ketoglutarate will be removed from the cycle by synthetic reactions and the isocitrate decarboxylation will be quantitatively less important than in the ammonium cells. But the overall rate of decarboxylation is the same whether nitrate or ammonium-N is added and, therefore, other decarboxylations must be of greater magnitude in the nitrate cells. These decarboxylations may be other decarboxylations in the tricarboxylic acid cycle, or they may be decarboxylations associated with an alternative oxidative pathway. If they are decarboxylations in the tricarboxylic acid cycle the balance between the various reactions of the cycle must differ in the ammonium and nitrate cells. The possible difference is depicted in Figure 2.

It is possible therefore, that reactions involving the C_4 acids are more important quantitatively in the nitrate cells than in the ammonium ones. It is interesting that Wolfe (1954) has shown that the blockage of nitrate reduc-
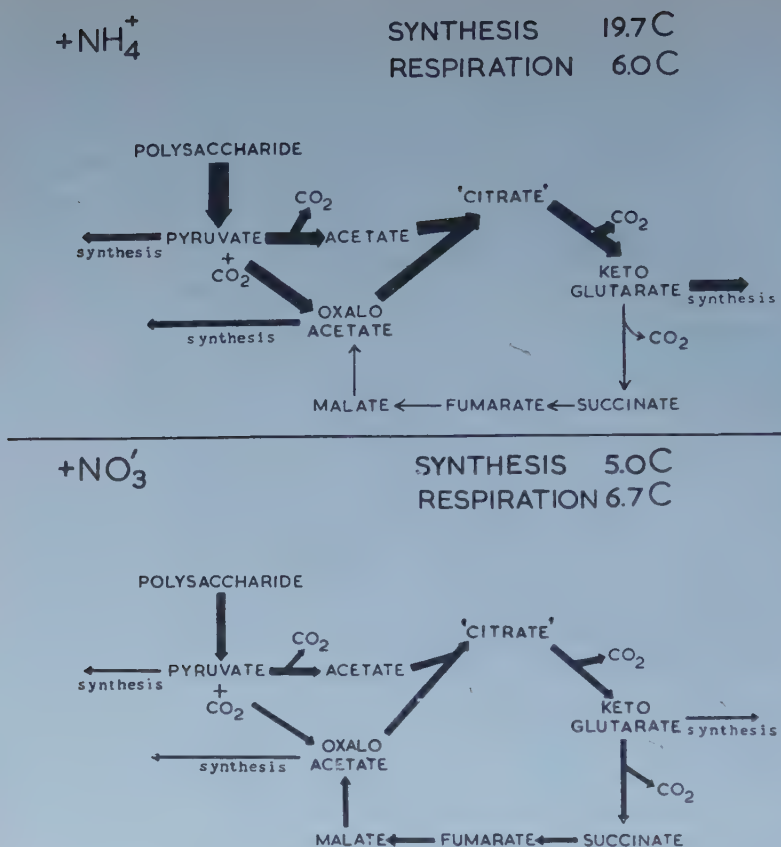
Figure 2. *Possible relative rates of reactions if carbohydrate is converted to amino-acids via the organic acids of the tricarboxylic acid cycle.* The thickness of the arrows represents the relative rates of the various reactions. 'Citrate' includes all the C_6 acids of the cycle. The figures for synthesis and respiration are taken from the preceding paper (Syrett 1956) and represent the number of carbon atoms involved in a given time.

tion in molybdenum deficient cells of Anabaena can be removed by the addition of fumarate whereas citrate is less effective and acetate has no effect at all.

In view of the large amount of carbon dioxide fixation which accompanies assimilation, it is surprising, at first sight, that cells in conventional War-burg vessels with caustic potash papers in the centre assimilate ammonium-N rapidly. However, application of the equations of Myers and Matsen (1955) shows that the concentration of carbon dioxide in solution in such vessels corresponds to a tension of about 2 mm. Calvin (1949) states that the dark fixation reactions of *Scenedesmus* are saturated at a carbon dioxide tension

of 0.5 mm. Presumably, even in a Warburg flask with caustic potash, respiration maintains a sufficiently high carbon dioxide concentration around the cells for assimilation to proceed unchecked.

Summary

The assimilation of ammonium-N by nitrogen-starved cells of *Chlorella vulgaris* in darkness is accompanied by much carbon dioxide fixation. This is expected if carbohydrate is converted to amino-acids via the organic acids of the tricarboxylic acid cycle. Less dark carbon dioxide fixation accompanies nitrate assimilation and this is in agreement with the lower rate of synthesis in the nitrate treated cells. Assuming that the tricarboxylic acid cycle operates in these cells. a possible difference in the balance of the reactions of the cycle between ammonium and nitrate cells is discussed.

References

Calvin, M.: The path of carbon in photosynthesis. VI. — J. Chem. Educ. 26: 639. 1949.
— Heidelberger, C., Reid, J. C.. Tolbert, B. M. and Yankwich, P. F.: Isotopic carbon. — New York: John Wiley & Sons, Inc. 1949.
Fowler, E. B. & Werkman, C. H.: Rôle of carbon dioxide in the bacterial synthesis of aspartate. — Arch. Biochem. & Biophys. 36: 365. 1952.
Greenberg, D. M.: Chemical pathways of metabolism. Vol. 2, Chap. 11. — New York: Academic Press Inc. 1954.
Myers, J. & Matsen, F. A.: Kinetic characteristics of Warburg manometry. — Arch. Biochem. & Biophys. 55: 373. 1955.
Ochoa, S.: Biosynthesis of dicarboxylic and tricarboxylic acids by carbon dioxide fixation. — Symposia of the Society for Experimental Biol. 5: 29. 1951.
Syrett, P. J.: The assimilation of ammonia and nitrate by nitrogen-starved cells of Chlorella vulgaris. III. Differences of metabolism dependent on the nature of the nitrogen source. — Physiol. Plant. 9: 28. 1956.
— & Fowden. L.: The assimilation of ammonia by nitrogen-starved cells of Chlorella vulgaris. III. The effect of the addition of glucose on the products of assimilation. — Physiol. Plant. 5: 558. 1952.
Wolfe, M.: The effect of molybdenum upon the nitrogen metabolism of Anabaena cylindrica. II. A more detailed study of the action of molybdenum in nitrate assimilation. — Ann. Bot. Lond. N.S. 18: 309. 1954.

The Cultivation *in vitro* of Tumor Tissues and Normal Tissues of *Picea glauca* [1]

By

JAKOB REINERT [2] and PHILIP R. WHITE [3]

Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine
(Received November 4, 1955)

Trees of the white spruce, *Picea glauca,* in certain restricted areas of the northeastern coast of North America are affected by large numbers of massive tumorous growths. Similar growths are reported on Sitka spruce in corresponding localities along the northwestern North American coast (Schwerin, 1917; Baxter, 1954), on spruces in swampy areas in Germany (Sprengel, 1936), and from certain scattered lake areas in Canada (Canadian National Park Service, 1953) but not from other continental areas. They do not appear to be "burls" of traumatic origin such as are commonly seen on so many species of trees. Their sharply and characteristically limited distribution and certain features of their gross and microscopic anatomy lead us to believe that they are true tumors (White and Millington, 1954 a, b). Because of the recognized similarity between malignant processes in animals and plants (White and Braun, 1942; White, 1949 b) and the advantages which plants hold in many respects for studies of malignancy (Riker and Hildebrandt, 1953; White and Millington, 1954 b) these tumors have been under study for some years. It has seemed desirable to establish, if possible, the

degree of their malignancy and the specific biochemical characteristics of the tissues involved.

One method of studying these questions, which has in the past proved fruitful, has been the cultivation of the affected tissues *in vitro* (White, 1939; White and Braun, 1942). Conifer tissues have not been extensively studied by this method. Ball (1950) has established slow growing continuous cultures of tissues isolated from young adventive shoots from burls of *Sequoia sempervirens.* Loewenberg and Skoog (1952), using tissues from embryos and young seedlings of *Pinus strobus,* established cultures on a nutrient supplemented with extracts of malt or of pine seeds. Tissues isolated from older branches were grown on this medium for short periods only. Wetmore and Morel (1951) report growing tissues from older branches of *Pinus strobus* on a chemically defined nutrient. Only in this last, unpublished work have tissues from the cambial region of mature trees such as those available to us from tumors on *Picea glauca* been grown on defined nutrients such as we have considered necessary for this work.

Research on this problem was first undertaken by one of us (P.R.W.) during the summers of 1948—50 at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine. Attempts to cultivate tissues isolated on White's nutrient (1943) which supports rapid growth of many plant tumor tissues (White, 1939, 1945; White and Braun, 1942) and of some normal tissues (White, 1953) failed, the cultures forming only a minimum of callus. Addition of i-inositol, Ca-pantothenate, biotin and naphthalene-acetic-acid (compare Morel, 1945, 1946; Gautheret, 1948 a), riboflavin, vitamin B_{12}, and the amino-acids of Madden and Whipple's formula (1943) used by White for the cultivation of animal tissues (1946, 1949 a) resulted in fair initial callus growth, but cultures regularly turned black and ceased to grow after a few weeks. Attempts to subculture the white initial callus failed, the explants promptly dying. Addition of ascorbic acid for a short period during the first 48 hours after excision, as recommended by Wetmore and Morel (1951) failed to overcome this blackening.

In 1951 the problem was transferred to the Roscoe B. Jackson Memorial Laboratory on a year round basis and was at that time placed in the hands of Dr. W. F. Millington as one of a series of approaches. In September, 1953, one of us (J.R.) undertook to continue this research as a major project. In this paper we wish to present the results of studies aimed at the *in vitro* cultivation of tissues isolated from tumors, from non-tumorous regions of tumor bearing trees, and from unaffected trees of *Picea glauca,* all tissues being isolated from mature trees.

Materials and Methods. — Material for cultivation was obtained as follows.

Where possible a tree or tumor not less than 8 inches in diameter was chosen. In the selected area four incisions were made with a saw or chisel blocking out a rectangle about 10 cm. on a side, the incisions penetrating to a depth of a centimeter or more into the wood. With the chisel the delimited block was removed, care being taken not to separate bark and wood at the cambium level. Throughout most of the year this presented no difficulty with normal wood in which the cambium is relatively firm except from May to August. Great care had to be exercised with tumor wood in which the cambium is moist for a much longer period. Cells capable of prompt growth were apparently present at all seasons.

The isolated blocks were brought into the laboratory in as nearly aseptic condition as possible. During those seasons when the cambium was moist, the blocks were sometimes separated at the cambium without any surface sterilization. The wood portion was discarded, the bark laid down cambium side up and a series of incisions made with a sterile scalpel 3—5 mm. apart and intersecting at right angles, to a depth of 2—3 mm., taking care that the cuts did not extend to the exposed margins of the block. A number of blocks 3—5 mm. on a side were thus laid out, which could be lifted out to a depth of 1 mm. or more and transferred to the nutrient, phloem side down. While this method was operationally simple the cambium was largely destroyed, growth could occur only from phloem cells, and the living surface was exposed to desiccation, oxidation, and trauma. Another method which proved more satisfactory was to surface sterilize the entire block by washing in 95 per cent alcohol several times and burning off the alcohol. The bark was then carefully cut away with sterile knives to within about 2 mm. of the cambium and any damaged tissue removed at one margin. Then a series of parallel incisions was made about 5 mm. apart, extending to this clean margin and penetrating about 2 mm. into the wood. Finally a second series of cuts was made parallel to the margin and blocks 5 mm square and about 4 mm. thick were removed and transferred to the nutrient, again phloem side down. These thus consisted of both phloem and xylem separated by undisturbed cambium, only the edge of the cambium being exposed throughout the circumference of the block. The cambium was thus protected. This proved by far the more satisfactory method and was used in most of this study.

Most plant tissue cultures have been grown on an agar substratum (Gautheret, 1942; White, 1943). In preliminary experiments Picea tissues made relatively unsatisfactory growth on this medium. In order to avoid this and to eliminate possible nutrient or adsorptive properties of agar, cultures for this study were grown in a liquid medium supported on filter paper (Whatman No. 1) using a modification (White, 1954) of a method first described by Heller (1949). Most nutrients were sterilized by autoclaving. Where relatively heat-labile substances (folic acid=pteroyl-glutamic acid; calcium pantothenate; glutamine; hypoxanthine, etc.) were included, these were sterilized separately by filtration through a Selas 03 porcelain candle and added to the nutrient aseptically. The pH of the nutrient, which is unbuffered, was about 4.8—4.9. Cultures were established in 25×150 mm. Pyrex test tubes provided with 10 ml. of nutrient and were kept in the dark at a temperature of $26° \pm 3°$ C.

The nutrient formula used in the beginning was one taken over from Millington (12 a). It consisted of White's nutrient solution (Table 1 A) (White, 1943), a group of amino acids (Table 1 B) (Modified from White, 1949 a) and a supplement of B-vitamins, asparagine and naphthalene-acetic-acid (Table 1 C).

Table 1.

A. *White's basic plant tissue nutrient.*				
sucrose	20,000.0 [1]		DL-valine	13.0
glycine	3.0		DL-isoleucine	10.4
nicotinic acid	0.5		DL-phenylalanine	5.0
pyridoxine	0.1		L-leucine	15.6
thiamin	0.1		L-tryptophane	4.0
$MgSO_4$	360.0		L-glutamic acid	14.0
Na_2SO_4	200.0		L-aspartic acid	6.0
$Ca(NO_3)_2$	200.0		L-proline	5.0
KNO_3	80.0		L-cystine	1.5
KCl	65.0		glycine	10.0
NaH_2PO_4	16.5		phenol red	5.0
$Fe_2(SO_4)_3$	2.5			

A. *White's basic plant tissue nutrient.*

sucrose	20,000.0 [1]
glycine	3.0
nicotinic acid	0.5
pyridoxine	0.1
thiamin	0.1
$MgSO_4$	360.0
Na_2SO_4	200.0
$Ca(NO_3)_2$	200.0
KNO_3	80.0
KCl	65.0
NaH_2PO_4	16.5
$Fe_2(SO_4)_3$	2.5
$MnSO_4$	4.5
$ZnSO_4$	1.5
H_3BO_3	1.5
KI	0.75

DL-valine	13.0
DL-isoleucine	10.4
DL-phenylalanine	5.0
L-leucine	15.6
L-tryptophane	4.0
L-glutamic acid	14.0
L-aspartic acid	6.0
L-proline	5.0
L-cystine	1.5
glycine	10.0
phenol red	5.0

C. *Supplement.*

i-inositol	100.0 [1]
Ca-pantothenate	0.1
ascorbic acid	0.1
folic acid (pteroylglutamic acid)	1.0
p-amino-benzoic acid	0.1
vitamin B_{12}	0.0015
choline chloride	10.0
riboflavin	0.1
L-asparagine	20.0
naphthalene acetic acid	0.05

B. *Amino-acid supplement.* [2]

L-lysine HCl	15.6 [1]
L-arginine HCl	7.8
L-histidine HCl	2.6
DL-methionine	13.0
DL-threonine	13.0

Results. On the nutrient given in Table 1 callus formation from both normal and tumor tissues proceeded well for about 2—3 weeks. Thereafter the pronounced darkening noted earlier regularly set in and growth diminished or ceased. If the white callus tissue was excised in an attempt to establish subcultures and the older tissues discarded, the change of color and cessation of growth occurred very rapidly, usually within 3—4 hours. Permanent cultures were not established in this nutrient.

The formula in Table 1 contains certain illogical features. *Para*-aminobenzoic acid is a precursor of folic acid which is already present in the nutrient. Glycine occurs in two places, once in White's nutrient (3 mg./l.) and again in the amino acid formula (10 mg./l.). In attempting to improve the nutrient *p*-aminobenzoic acid and the 3 mg./l. portion of glycine were omitted, retaining the larger amount in the amino acid supplement. This supplement was also changed by reducing the cystine concentration to 1.25 mg./l. and the phenol red to 0.02 mg./l. Further improvement was sought

---

[1] All concentrations given are in mg./l. of the final nutrient.

[2] The concentration of amino-acids found suitable for plant tissues is about one-tenth that used for animal cells.

Table 2. *Effects of various substances tested as additions to the nutrient of Table 1 or substituted for NAA therein.* Concentrations in mg./l.

Growth promoting		Without effect		Retarding	
hypoxanthine	25.0	MoO_2	0.01	Na-nucleate	5.0
glutamine	50.0	$CoCl_2 \cdot 6H_2O$	0.05	adenine	40.0
biotin	0.01	IAA	0.05	$CuSO_4$	0.05
2,4-D	0.05	yeast extract	10.0		
malt extract	100.0				

by addition to rather than deletion from this modified nutrient. Substituting 2,4-dichlorophenoxyacetic acid (2,4-D, 0.05 mg./l.) for NAA and adding hypoxanthine, glutamine and biotin, each improved the growth of spruce cultures when tested separately (Table 2). Added together, they produced a 70 per cent lengthening of the mean survival time of the cultures. However, blackening of the tissue and cessation of growth set in after 6 to 7 weeks.

The parallelism between the onset of pigmentation and the cessation of growth suggested a causal relationship between growth and the activity of pigment forming, copper containing enzymes (phenoloxidases). Such a relation has already been suggested for other tissues by Wetmore and Morel (1951) in connection with the destruction of auxins and by Jacquiot (1947) in considering the role of tannins. Experiments were therefore undertaken with the metal component (copper), the substrate (tyrosine), and with inhibitors of phenoloxidases; glutathione and ascorbic acid at very high concentrations (10—100 mg./l.), Na-diethyldithiocarbamate and Na-ethylxanthate, also in the same concentration range. The medium of Table 1, modified as described above was used as a control.

With 40 mg./l. tyrosine and 0.01 mg./l. $CuSO_4$ added to the nutrient, cultures grew more rapidly than the controls during the first three weeks. Thereafter, particularly when the newly formed callus was excised to set up subcultures, they turned brown as before and ceased to grow. Without copper but with either tyrosine or one of the phenoloxidase inhibitors the cultures continued to grow much longer. They did not turn brown until about 2 months after excission and if clean white callus was removed before this time, the new tissue survived without browning and continued to grow. Cultures established on a nutrient containing Na-ethylxanthate or diethyl-dithiocarbamate died after a single such subculturing. Those with glutathione or ascorbic acid died after the second or third subculturing. But those with tyrosine continued to grow, forming flat crustose masses (Fig. 1), which could be propagated further without difficulty. They are now (July, 1955) in their seventeenth passage and are about a year and a half old.

With this experience, using this medium or a somewhat modified one

Figure 1. *Well established cultures on a medium containing 2,4-D.* At the left, normal tissue, about 9 months old, in the 8th passage. At the right tumor tissue, about 11 month old, in the 10th passage. Both are growing rapidly. Note the greater pigmentation of the tumor tissue. Note also the small, isolated, rapid growing masses above and below the main culture at the left. These have arisen from small groups of cells which have separated from the main cultures. $\times 1\,^{1}/_{2}$.

(Table 3) but always containing tyrosine, a number of strains of tissue have been established. When, in addition to tyrosine there was supplied gluta-thione or ascorbic acid at high concentration (10 mg./l.), growth, especially in tumor cultures, was better than in the control nutrient during the first 8—12 weeks but thereafter it was slower than in nutrients without these two components.

Another factor which is probably influenced by the same enzyme system as is tyrosine metabolism, and which responds differently in normal and tumor tissues, is the auxin system. As figures 2 and 3 show, if 2,4-D is replaced by IAA growth can be obtained only if very high concentrations of IAA are used (1—10 mg./l., Figure 2) or if, in addition to lower con-centrations of auxin (0.01 mg./l.) there are added glutathione, diethyldithio-

Figure 2. *The effect of different levels of indole-acetic-acid on growth of primary explants, 49 days after ex-cission.* All nutrients contained Vita-min B_{12} and folic acid (pteroylglu-tamic acid). Top, 5×10^{-8} g/ml. IAA; left 10^{-6}; right 10^{-5}. Note that at 5×10^{-8} very little callus has formed while at 10^{-6} (left) and 10^{-5} (right) callus has developed extensively. $\times 3$.
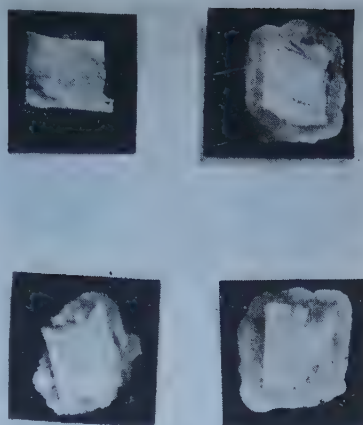
Figure 3. *The effect of indole acetic acid, with and without IAA inhibitors, 35 days after excission.* Upper left 5×10^{-8} g/ml. of IAA. Upper right, the same plus 10^{-4} g/ml. of diethyldithiocarbamate. Lower left, same as upper left plus 10^{-4} Na-ethylxanthate. Lower right, same as upper left plus 10^{-5} glutathione. The presence of any one of these three SH compounds results in extensive formation of callus. $\times 3$.

carbamate etc. (Figure 3). This is regularly true for tumor tissues. It is usually true for normal tissues as well, but in exceptional cases normal tissue will produce extensive callus even at low concentrations of IAA (Figure 4). As in earlier experiments, the growth promoting effect of inhibitors persisted mainly during the first 5—10 weeks. Thereafter cultures on a medium with these combinations and with all concentrations of IAA lower than 10 mg./l. ceased to grow.

Once it was established that all factors necessary for continued growth of Picea glauca tissues were present in the admittedly rather complex medium (compare Tables 1 and 3) it was possible to approach the problem of simplifying this medium. Tests of the vitamin group showed that vitamin B_{12} was without effect on growth of normal tissue. Of four tumor strains derived

Figure 4. *Growth of normal tissue* (bottom row) *and tumor tissue* (top row) *on media containing indole acetic acid* (all cultures, 5×10^{-8} g/ml.) *either with* (left) *or without* (right) 10^{-4} g/ml. of *Na-diethyldithiocarbamate.* Note that callus is better developed in the presence of the SH compound (left) and that this is especially true in the tumor tissue (upper left). $\times 3$

Table 3. *Nutrient Supplement to replace Supplement C of Table 1.*

i-inositol	100.0 [1]	L-asparagine	20.0
Ca-pantothenate	0.1	glutamine	50.0
biotin	0.01	hypoxanthine	25.0
ascorbic acid	0.1	2,4-D	0.05 [2]
riboflavin	0.1	L-tyrosine	40.0
choline chloride	10.0		

[1] Concentrations in mg./l.

[2] 2,4-D can be replaced by IAA at 10 mg./l.

from different trees, one can likewise be maintained without B_{12} while the other three can be subcultured only when the medium contains this vitamin. Folic acid (pteroylglutamic acid) at the concentration used (1 mg./l.) has a slight but definite retarding effect. It may conceivable be stimulating at lower concentrations not yet tested. Growth will continue without biotin but its omission causes a clear decrease in growth rate. It should therefore be retained, as Morel and Gautheret found to be the case with tissues of Cratae-gus (1946) and Salix (1948 a). Tumor wood consistently failed to grow in the absence of ascorbic acid. Normal tissue usually requires a low level of ascorbic acid although two strains have been established which grow well in its complete absence. It has not been possible to reduce the number of amino acids used. Although both tyrosine and phenylalanine, and both glutamine, asparagine and their corresponding acids are present in the formula used, experiments in which phenylalanine, glutamic acid, or aspartic acid were omitted led to satisfactory growth for 10—12 weeks, but followed by retarda-tion and final cessation of growth. Apparently all of these ingredients are essential for continued growth.

Our experiments so far indicate no likelihood of simplifying the nutrient supplement given in Table 3, which must be added to the modified ingre-dients of Table 1 A and 1 B.

On this nutrient, tissues from normal spruce wood can be regularly established as rapid growing strains. This is true of tissue taken from both unaffected trees and from normal regions of tumor bearing trees. The pre-sence of a tumor does not appear to effect the viability, the growth charac-teristics or the nutrient requirements of adjacent unaffected normal wood. Callus from normal cambium is regularly white and although the growth pattern may be altered by changes in nutrient, any medium which induces browning concommitantly results in death. Tumor tissue, on the other hand is much more erratic in behavior. While most cultures succeed on this nutrient there are many failures. The callus formed tends to be irregular and sometimes deeply pigmented (Figure 1, right) yet on suitable media there

Figure 5. *Different growth habits of tumor tissues.* Upper pair, two sister cultures on a nutrient containing 10^{-5} g/ml. IAA, fourth passage. Both grew as compact, woody, ovoid masses. When removed from the substratum they appear as inverted bowls, only the edges being in contact with the nutrient.
Lower pair, two sister cultures on a nutrient containing 2,4-D, 562 days. Neither is compact and ovoid as above. That at the right is friable and heavily pigmented, that at the left thin, crustose and with much unpigmented growth. $\times 1\,^1/_2$.

are usually scattered areas of unpigmented callus from which rapidly growing and often persistently nonpigmented subcultures can be isolated. Moreover, the growth pattern on a given nutrient formula is not stable. Figure 5 top pair, shows two cultures from a single strain of tumor tissue. These are pigmented on the right side but with white callus masses on the left. Both are compact, woody, and upon inversion appear as bowls, growth having occurred largely at the substrate-tissue-air three-phase contact. This type of growth has been previously noted as characteristic of cultures of Nicotiana tissue (White, 1939). Figure 5 bottom pair shows two other tumor cultures, both from a single isolation. These have been in culture for 562 days on a nutrient containing 2,4-D. That on the right is highly friable, not at all solid, resembling in this respect de Ropp's P_{II} strain of sunflower tissue (deRopp, 1947) and is deeply pigmented except for certain small areas at the left. Once a culture of normal tissue has taken on this appearance it cannot be revived, yet this one is quite viable. The one at the left is flat, crustose as described for tissues of Salix and Crataegus by Gautheret and Morel, and with very little pigmentation. All three of these growth types appear repeatedly and all three seem to be equally viable and may sporadically change from one type to another without evident reason. Such instability has not been noted with normal tissue and appears to be a definite difference between tumor and normal materials, in addition to the differences in response to growth substances.

Discussion. Our initial efforts to develop a suitably balanced nutrient capable of supporting permanent, rapid growing cultures of these tissues were somewhat hampered by the rather complicated medium with which the experiments were begun. The great number of components made it impractical to reexamine for optimal concentrations and for growth promoting or growth restricting properties even a fraction thereof. For this reason, after eliminating or reducing certain unnecessary or possibly damaging components such as *p*-amino-benzoic acid, glycine etc., attention was concentrated on two matters: first, factors which might increase protein synthesis and second, factors which might restrict the activity of certain enzymes (phenoloxidases and indole acetic acid oxidases) which appear to be especially active in traumatized tissue (Reinert, 1955; Wetmore and Morel, unpublished data). Adding substances whose effects in increasing growth of protoplasm are well recognized (2,4-D, glutamine, hypoxanthine. Compare Rebstock et al, 1952; Steward and Street, 1946; Gale and Folke, 1953) increased the period of survival of the cultures and provided a starting point for definitive experiments to eliminate the growth restricting action of the phenoloxidases. The successful subculturing of the spruce tissues, confirmed the suitability of this approach. We must, however, point out that our results do not yet permit a firm conclusion as to whether, for example, the growth restricting effect of phenoloxidases arises from a rapid destruction of tyrosine, from the formation of toxic intermediary products such as quinones, or both. The use of enzyme inhibitors likewise does not permit us to clearly distinguish whether IAA-oxidases occur in the tissues in addition to the phenoloxidases, nor does it show definitely whether there is a connection between the degradation of growth substances and the activity of phenoloxidases as Wetmore and Morel (1951) suggested

Our results with adenine and hypoxanthine differ from those of Skoog and Tsui, (1948) with tobacco pith but are in agreement with the findings of Nickell et al (1950) on virus tumor tissues of *Rumex acetosa*. The fact that biotin enhanced growth of spruce tissues in these and in earlier experiments (P.R.W) accords with the results of Morel (1945) and Gautheret (1948 a). It remains to be determined whether the growth retarding effect of folic acid is due to the use of too high a concentration or whether this vitamin has an inhibiting effect *per se*.

In the course of this investigation differences were noted between normal and tumor tissues as regards growth rates, nutrient requirements and degrees of variability. These differences were consistent in spite of the fact that both normal and tumor tissues wer taken from a number of different trees, hence from material which was not genetically homogenous. Freshly isolated tumor tissues, when they grew at all regularly began to form callus two to three

days before there was any trace of growth on simultaneously isolated normal tissues, either from the same tree or from other trees. Yet failure of growth was more frequent with tumor than with normal tissues. Moreover, once established, normal tissues were much more consistent in behavior than were tumors. With normal tissues differences in constitution of nutrient regularly produced consistent differences in pigmentation, rapidity of growth etc. This was not the case with tumor tissues in which some isolations behaved alike while others differed widely in appearance and in growth rate. Drastic changes in nutrient such as omission of riboflavin and choline, or of various amino acids (unpublished), produced evident results much later with tumor cultures than with normal, and tumor cultures continued to grow much longer (up to two months) on such deficient nutrients than did normal tissues from the same tree. This suggests that tumor tissues may possess a somewhat higher capacity to synthesize these substance than do normal tissues, a behavior comparable to that reported for crown-gall tumor cells with respect to the auxins (Gautheret, 1948 b; de Ropp, 1947 b). Their reaction toward vitamin B_{12}, on the other hand, was the reverse. With one exception out of a great number, tumor tissues could be subcultured over long periods only when the medium contained vitamin B_{12}, a substance which was not necessary for the growth of normal tissues. Here the synthetic capacity of the tumor appears to be less than the normal and the one exception noted would suggest that in this one case the tissue isolated from what appeared to be a tumor was in some respect more nearly "normal" than its location would lead us to expect. This reaction to vitamin B_{12} is the more striking since B_{12} has not previously been reported to be required for growth of any higher plant but only for certain bacteria and algae (compare Darken, 1953).

We have not yet examined the question whether these tumors are truly malignant in the sense of being capable of artificial metastasis (producing overt tumors upon transplantation into otherwise healthy trees) as are so many sterile crown-gall tissues. We do now have, however, an effective method of producing considerable quantities of rapid growing, sterile, disorganized tissue of known origin, such as has in crown-gall proved useful in the study of malignancy and its nature.

Summary

Tissues of *Picea glauca* excised from the cambial region of the massive epiphytotic tumors characteristic of this species in certain regions, from non-tumorous portions of tumor-bearing trees and from trees not carrying evident

tumors have been cultivated *in vitro* as continuous rapid growing cultures maintained on filter paper saturated with a complex defined liquid nutrient. The rapid blackening which regularly follows trauma of tissues with an active polyphenoloxydase system, as is true of many tree tissues, and which results in cessation of growth, could be overcome either by addition of tyrosine to the nutrient or by the use of phenoloxydase inhibitors such as diethyl-dithiocarbamate. These inhibitors were, however, only temporarily effective. Only tyrosine permitted continued growth. The auxine system appears to be closely associated with the polyphenoloxidase system and indole acetic acid was effective only at excessively high concentrations, being better replaced by 2,4-D.

Tumor tissues, when they grew at all, consistently began growing more promptly than did normal tissues. There were, however, more frequent failures of tumor cultures and their responses to changes in nutrient or culture conditions were much more erratic than in the case of normal tissues. The presence of tumors did not effect the behavior of non-tumor tissues of tumor-bearing trees. Tumor tissues appeared to require Vitamin B_{12} which is not required by normal tissues.

References

1. Ball, E.: Differentiation in a callus culture of Sequoia sempervirens. — Growth 14: 295. 1950.
2. Baxter, Don V. (Personal communication). 1954.
3. Canadian National Park Service. (Personal communication). 1953.
4. Darken, Marjorie A.: Production of vitamin B-12 by microorganisms and its occurrence in plant tissues. — Bot. Rev. 19: 99. 1953.
5. Gale, E. F. & Folkes, J. P.: Protein synthesis enhanced by purine derivatives. — Biochem. Journ. 53: 483. 1953.
6. Gautheret, R. J.: Manuel technique de culture des tissus végétaux. — Masson, Paris. 1942.
7. — Sur la culture indefinie des tissus de Salix capraea. — C. r. Soc. Biol. 142: 807—808. 1948 a.
8. — Action de l'acide indole-acétique sur le développement de 3 types de tissus de Scorsonère: tissus normaux, tissus de Crown-Gall et tissus accoutumés à l'hétero-auxine. — C. r. Soc. Biol. 142: 774. 1948 b.
9. Heller, R.: Sur l'emploi de papier filtre sans cendres comme support pour les cultures de tissus végétaux. — C. r. Soc. Biol. 143: 335. 1949.
10. Jacquiot, C.: Effet inhibiteur des tannins sur le développement des cultures in vitro du cambium de certains arbres forestiers. — C. r. Adad. Sci. Paris. 225: 434. 1947.
11. Loewenberg, J. R. & Skoog, F.: Pine tissue cultures. — Physiol. Plant. 5: 33. 1952.
12. Madden, S. C., Carter, J. R., Kattus, A. A., Miller, L. L. & Whipple, G. H.: Ten amino-acids essential for plasma protein production effective orally or intravenously. — J. Exp. Med. 77: 277. 1943.
12a. Millington, W. F. (unpublished data).

13. Morel, G.: Recherches sur le développement de fragments de lianes cultivés in vitro. — Bull. Soc. Bot. France. 92: 98. 1945.

14. — Action de l'acide pantothénique sur la croissance des tissus d'Aubépine cultivés *in vitro.* — C. r. Acad. Sci. Paris 223: 166. 1946.

15. Nickell, L. G., Greenfield, P. & Burkholder, P. R.: Atypical growth of plants. III. Growth responses of virus tumor of Rumex to certain nucleic acid compounds and related compounds. — Botan. Gaz. 112: 42. 1950.

16. Rebstock, T. L., Hamner, C. L., Hamner, C. D., Ball, C. D. & Sell, H. M.: Effect of 2,4-dichlorophenoxyacetic acid on proteolytic activity of red kidney bean plants. — Plant Physiol. 27: 639.

17. Reinert, J.: Über die Bedeutung kupferhaltiger Enzyme für die Kultur von Koniferengewebe in vitro. — Naturwiss 1: 18—19. 1955.

18. Riker, A. J. & Hildebrandt, A. C.: Some factors associated with diseased growth in plants. — In W. E. Loomis: Growth and Differentiation in plants, p. 393. Iowa State College Press, Ames, Iowa. 1953.

19. de Ropp, R.: The isolation and behavior of bacteria-free crown gall tissue from primary galls of Helianthus annus. — Phytopath. 37: 201. 1947 a.

20. — The response of normal plant tissues and of crown-gall tissues to synthetic growth hormones. — Am. J. Bot. 34: 53. 1947 b.

21. von Schwerin, F.: Merkwürdige Stammbildungen der Sitka Fichte. — Mitt. Deutsch. Dendrol. Gesell. 26: 227. 1917.

22. Skoog, F. V. & Tsui, C.: Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. — Am. J. Bot. 35: 782. 1948.

23. Sprengel, F.: Über die Kropfkrankheit an Eiche, Kiefer und Fichte. — Phytopath. Ztschr. 9: 583. 1936.

24. Steward, F. C. & Street, H. E.: The soluble nitrogen fractions of potato tubers; the amides. — Plant Physiol. 21: 155. 1946.

25. Wetmore, R. H. & Morel, G. (Personal communication). 1951.

26. White, P. R.: Potentially unlimited growth of excised plant callus in an artificial nutrient. — Amer. J. Bot. 26: 59. 1939.

27. — A Handbook of Plant Tissue Culture. — The Ronald Press, N.Y. 1943.

28. — Metastatic (graft) tumors of bacteria-free crown-galls on Vinca rosea. — Amer. J. Bot. 32: 237. 1945.

29. — Cultivation of animal tissues in vitro in nutrients of precisely known constitution. — Growth 10: 231. 1946.

30. — Prolonged survival of excised animal tissues in vitro in nutrients of known constitution. — J. Cell. Comp. Physiol. 34: 221. 1949 a.

31. — Les tumeurs végétales et le problème de l'etiologie cancéreuse. — Ann. Biol. 26: 745. 1949 b.

32. — A comparison of certain procedures for the maintenance of plant tissue cultures. — Am. J. Bot. 40: 517. 1953.

33. — The Cultivation of Animal and Plant Cells. — The Ronald Press, N.Y. 1954.

34. — & Braun, A. C.: A cancerous neoplasm of plants. Autonomous bacteria-free crown-gall tissue. — Cancer Res. 2: 597. 1942.

35. — & Millington, W. F.: The distribution and possible importance of a woody tumor on trees of the white spruce, Picea glauca. — Cancer Res. 14: 128. 1954 a.

36. — The structure and development of a woody tumor effecting Picea glauca. — Amer. J. Bot. 41: 353. 1954 b.

Le développement in vitro des colonies prothalliennes de *Gymnogramme calomelanos* (Filicinée Polypodiacée) en présence de mélanges binaires d'acides aminés aliphatiques. I. Morphologie générale et accroissement pondéral des cultures

Par

I. Sossountzov

Laboratoire de Botanique, Sorbonne, Paris
(Reçu le 20 Novembre 1955)

Dans un travail précédent, il a été montré que, pourvues en azote aminé, les colonies prothalliennes aseptiques de la fougère *Gymnogramme calomelanos* peuvent proliférer jusqu'au 3e (alanine et valine) ou jusqu'au 6e repiquages (glycocolle, leucine, sérine). Des cinq acides aminés utilisés comme source unique d'azote, seul le glycocolle permet une croissance normale des colonies dans d'étroites limites de concentration (1,5 à 5 mM) (1); avec les autres acides aminés, les cultures deviennent aberrantes («masse nécrosée centrale» couverte d'un mince tapis de prothalles vivants) (2). Dans la présente note seront exposés les résultats obtenus entre 1949 et 1955 avec les colonies prothalliennes de *G.c.* pour lesquelles l'appoint en azote était dû aux cinq amino-acides précités utilisés en mélanges deux-à-deux.

Techniques

Les techniques de culture ont été décrites antérieurement (1). Les colonies prothalliennes ont proliféré sur le milieu de Knop normal glucosé à 2 %, privé d'azote minéral et additionné d'azote aminé. Ce dernier a été fourni sous forme des mélanges suivants: glycolle (glycine)+DL-α-alanine, glycocolle+DL-valine, glycocolle+L(—)-leucine, glycocolle+DL-sérine; alanine+valine, alanine+leucine, alanine+sérine;

valine+leucine, valine+sérine; leucine+sérine. Les observations ont été faites au 45e jour suivant celui du repiquage; pour chaque combinaison de concentrations, elles ont porté sur 10 à 24 cultures. Les colonies-témoins ont proliféré sur le milieu de Knop normal glucosé (10,94 milliatomes grammes d'azote NO_3^-).

Données expérimentales

A. *Nombre de repiquages supportés par les cultures*

Comme il a été dit ci-dessus, lorsqu'on les utilise *isolément,* seuls le glyco-colle, la sérine et la leucine permettent aux cultures d'atteindre le 6e repi-quage; avec l'alanine et la valine, ces mêmes cultures ne survivent pas au 4e repiquage. Cependant, lorsqu'à chacun de ces deux acides aminés, on adjoint du glycocolle, de la leucine ou de la sérine, leur toxicité s'atténue: les cultures peuvent alors atteindre le 4e ou le plus souvent le 6e repiquages.

En désignant chacun des acides aminés utilisés par son initiale, voici jusqu'à quel repiquage (en abrégé: R) leurs combinaisons binaires permettent la croissance des prothalles:

$$\left.\begin{array}{l}G\\L\\S\end{array}\right\} 6^e\ R \qquad \left.\begin{array}{l}G+A\\G+L\\G+V\\G+S\end{array}\right\} 6^e\ R \qquad \left.\begin{array}{l}A+L\\A+V\\A+S\end{array}\right\} 6^e\ R \qquad L+V-3^e\ R \qquad V+S-4^e\ R$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad L+S -6^e\ R$$

$$\left.\begin{array}{l}A\\V\end{array}\right\} 3^e\ R$$

$\underbrace{}$ acides aminés utilisés *isolément*

$\underbrace{}$ acides aminés en *mélanges binaires*

La toxicité propre à chaque acide aminé est donc bien supprimée ou atténuée lorsqu'on lui adjoint un autre acide aminé.

B. *Concentrations maxima tolérées par les cultures*

Si l'on compare les valeurs des concentrations maxima permettant aux cultures d'atteindre le 45e repiquage, on constate que, lorsque les acides aminés sont employés en mélange, ces valeurs sont moins discrètes que lors-que ces mêmes acides sont utilisés isolément. Par exemple, au 6e repiquage, les cultures ne tolèrent que des concentrations en leucine inférieures à 5 mM; mélangée à un autre acide aminé, par exemple l'alanine, la leucine permet la prolifération prothallienne jusqu'à 7 à 8 m.M. L'effet toxique des fortes concen-trations en acide aminé est donc atténuée par l'adjonction d'un autre acide aminé.

La concentration maxima tolérable varie d'ailleurs avec celle de l'amino-acide accompagnateur (figures 3 et 4). Elle diminue en général à mesure
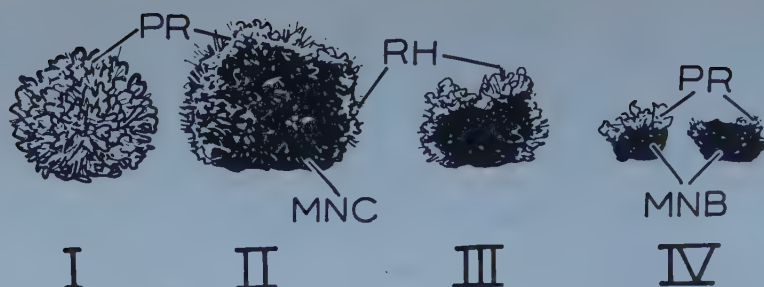
Figure 1. *Aspect schématique des colonies prothalliennes de 45 jours en section verticale.* I: *Culture normale.* Il n'y a pas de parties nécrosées. II à IV: *Cultures aberrantes.* II — culture à «masse nécrosée centrale» (MNC) couverte de prothalles vivantes (PR). Nombreux rhizoïdes (RH). IV — deux cultures à «masse nécrosée basale» (MNB) couverte seulement à sa partie supérieure de prothalles vivants (PR). A gauche, le tapis prothallien vivant est continu, à droite il est discontinu et laisse apparaître des affleurements de la «masse nécrosée basale» (MNB). III — type intermédiaire entre les deux précédents.

que celle de l'autre acide augmente. Exemple: la concentration en glycocolle permettant la croissance prothallienne est au maximum de 11 mM à condition que la teneur en alanine soit de 1 mM. Si cette teneur augmente, la concentration maximum en glycocolle diminue: ainsi, cette dernière est de 10 mM quand la concentration en alanine est de 2 mM; elle tombe à 9 mM avec 3 ou 4 mM d'alanine, etc. (3). C'est là une nouvelle manifestation de l'antagonisme entre acides aminés déjà mentionnée plus haut.

C. *Cultures du dernier repiquage possible*

I. *Morphologie générale des cultures*

Quelle que soit la composition du mélange binaire en acides aminés et quelles que soient les concentrations de ces derniers, les colonies prothalliennes observées présentent toutes une partie interne ou basale nécrosée couverte d'un tapis, généralement très mince, de prothalles vivants verts ou jaunâtres. La figure 1 présente schématiquement ces types de culture *aberrants*: cultures à «masse nécrosée centrale», cultures à «masse nécrosée basale», forme intermédiaire. Il apparait donc que l'atténuation de la toxicité des amino-acides lorsqu'ils sont en mélange n'est pas suffisante pour assurer aux colonies prothalliennes une croissance *normale,* telle qu'on l'observe par exemple sur les cultures-témoins (milieu de Knop), c'est-à-dire une croissance sans apparition de zones nécrosées au 45e jour suivant celui du repiquage considéré (fig. 1).

La figure 2 montre qu'en gros le type à «masse nécrosée centrale» s'observe
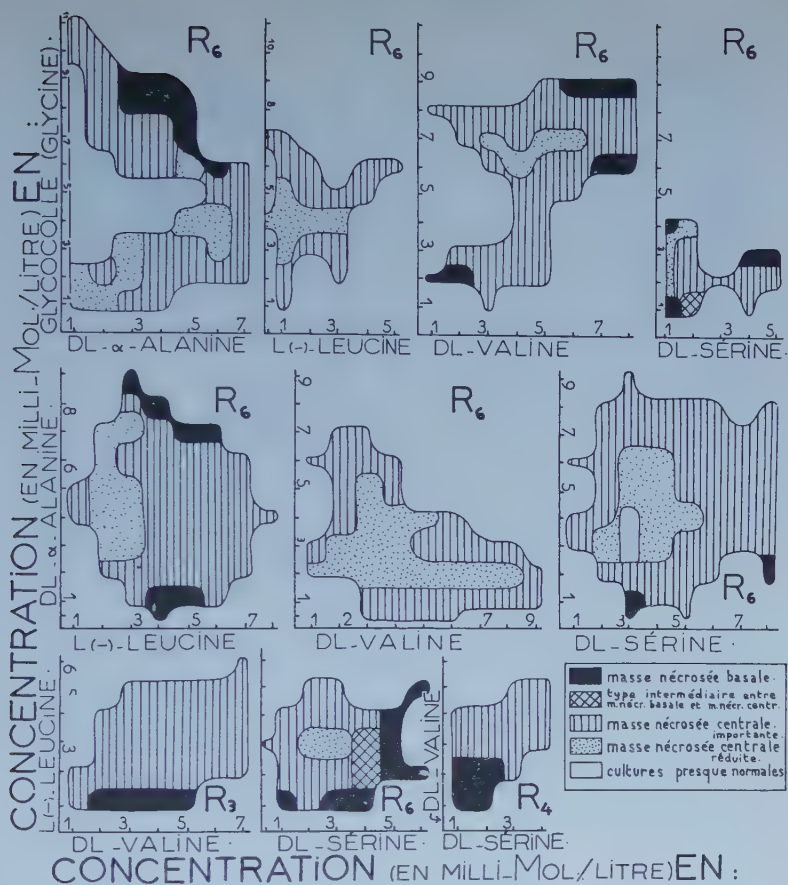
Figure 2. *Répartition des types de cultures de 45 jours sur des milieux à concentrations variables en deux acides aminés.* Les milieux nutritifs situés en dehors des zones délimitées sur les graphiques ne permettent pas la croissance des prothalles jusqu'au 45e jour.

en présence de concentrations moyennes en azote, alors que le type à «masse nécrosée basale» correspond aux concentrations en azote extrêmes, soit les plus faibles, soit les plus élevées. Seuls les mélanges glycocolle + alanine et alanine + sérine permettent aux cultures de se développer quasi-normalement («masse nécrosée centrale» à peine marquée au 45e jour).

En ce qui concerne le glycocolle qui, utilisé *seul,* permet la croissance normale des cultures (entre 1,5 et 5 mM) (1), on constate que, *mélangé* avec l'un des quatre autres acides aminés, il n'autorise plus qu'une croissance aberrante (formation d'une «masse centrale nécrosée» plus ou moins importante). Le glycocolle atténue donc bien la toxicité des autres acides aminés, mais ces derniers, en quelque sorte par compensation, le privent de son action favorable sur la croissance prothallienne. Ainsi l'état de toxicité moyenne qui s'établit entre le glycocolle et par exemple

13
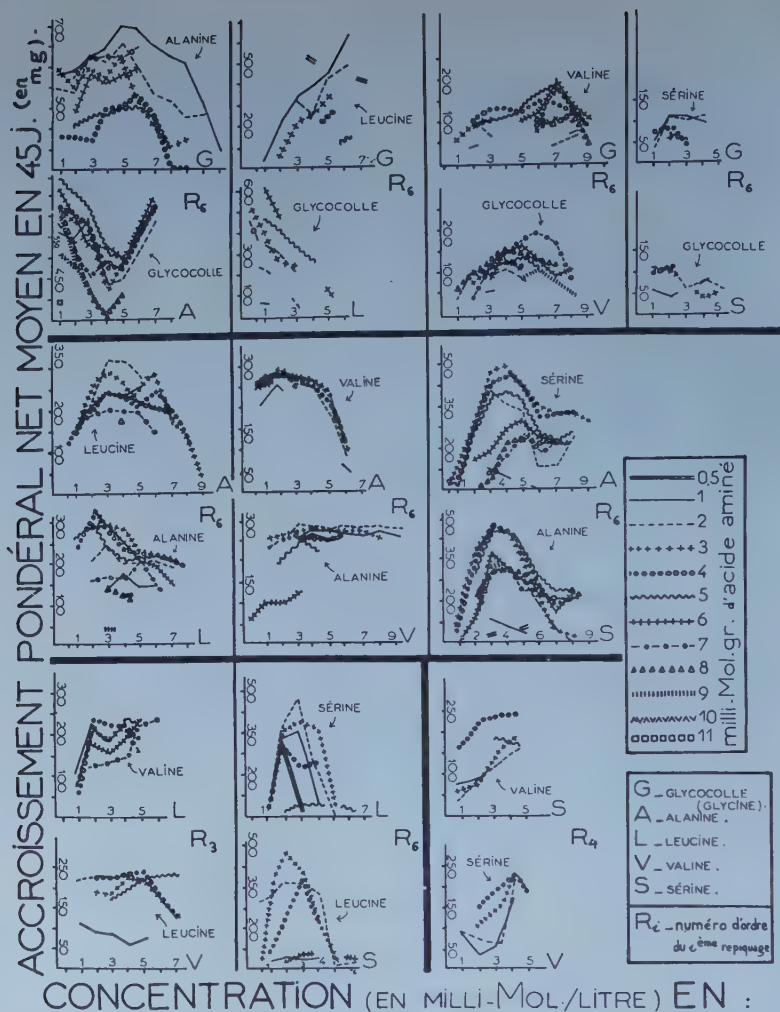
Figure 3. *Accroissement pondéral des colonies prothalliennes de 45 jours sur des milieux à concentrations variables en deux acides aminés.* En ordonnée — les valeurs en mg du poids net moyen des cultures de 45e jour. En abscisse — concentrations, en milli-mole, des acides aminés associés deux-à-deux. Voir aussi les courbes de la figure 4.

l'alanine, s'il permet aux cultures d'atteindre le 6e repiquage (malgré la présence d'alanine), leur autorise seulement une croissance aberrante (malgré la présence du glycocolle).

II. *Accroissement pondéral des cultures*

L'accroissement pondéral est exprimé en *poids net* (poids des prothalles débarrassés du liquide nutritif qu'ils immobilisent entre eux).
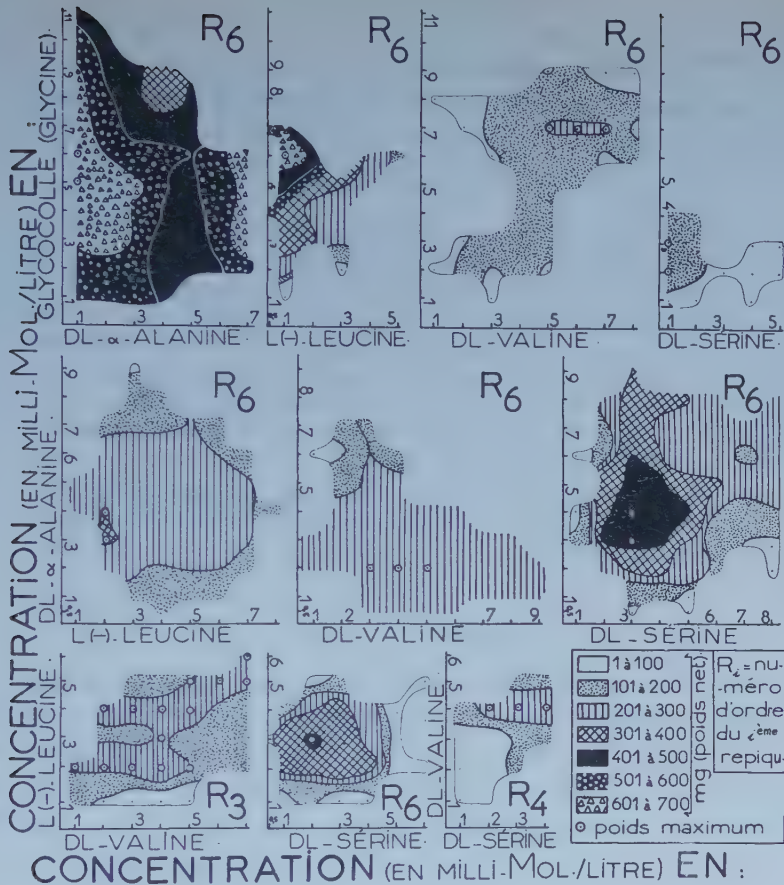
Figure 4. *Courbes de la figure 3 représentées en projection orthogonale.* Sur chaque milieu permettent la croissance prothallienne a été élevé un segment de droite perpendiculaire au plan de la figure et de longueur proportionnelle au poids net moyen (en mg) des cultures proliférant sur ce même milieu. Les sommets des segments d'égale hauteur ont été ensuite reliés par des courbes (lignes isobariques) dont les projections orthogonales sont représentées sur la figure. Les milieux nutritifs situés en dehors des courbes ne permettent pas la croissance des prothalles jusqu'au 45e jour.

On voit sur les figures 3 et 4 que seules les associations glycocolle + alanine, glycocolle + leucine, alanine + sérine et leucine + sérine permettent aux cultures d'atteindre des poids élevés. Les autres associations sont moins favorables à leur croissance.

Si l'on examine les courbes de la figure 3, on s'assure que, dans de nombreux cas, elles sont en forme de cloche à mesure qu'augmente la concentration de l'acide aminé considéré, et cela quelle que soit la concentration de

l'acide aminé qui lui est associé. En d'autres termes, la croissance maxima est obtenue en présence de concentrations moyennes en azote. Sur la figure 4, ce résultat se traduit par le fait que les milieux optimaux (marqués d'un cercle pointé) (croissance maxima) se situent dans les parties internes des zones délimitées par les courbes isobariques.

Quelques associations se comportent toutefois différemment, en particulier l'association glycocolle+alanine: lorsque la concentration en glycocolle augmente, la courbe des accroissements pondéraux est en cloche à maximum, indépendamment de la teneur des milieux en alanine (fig. 3, en haut à gauche); mais si l'on considère les variations de la teneur en alanine, on voit que la courbe des accroissements est en U, indépendamment de la richesse des milieux en glycocolle. Sur la fig. 4, cela se traduit par l'existence de deux zones de croissance rapide, l'une au niveau des plus faibles, l'autre au niveau des plus fortes concentrations en alanine; entre les deux se situe une zone de croissance plus lente.

De même, pour l'association glycocolle+leucine, le maximum de croissance s'observe en présence des teneurs en glycocolle les plus élevées combinées aux teneurs en leucine les plus faibles.

III. *Comparaison avec les cultures-témoins*

Bien qu'en mélange les amino-acides soient relativement peu nocifs pour les prothalles, en aucun cas ils ne présentent une valeur nutritive voisine de celle du milieu-témoin (milieu de Knop normal): cultures aberrantes en présence d'azote aminé, cultures-témoins normales; poids net moyen généralement moins élevé avec l'azote aminé qu'avec l'azote NO_3^- (environ 470 mg au 6e repiquage); cultures-témoins pouvant être repiquées indéfiniment, cultures à azote aminé, dans quelques cas, repiquables un nombre limité de fois.

Résumé

Vis-à-vis du développement in vitro des colonies prothalliennes de Gymnogramme, le glycocolle (glycine), la DL-α-alanine, la DL-valine, la L(—)-leucine et la DL-sérine sont beaucoup plus toxiques lorsqu'ils sont utilisés isolément comme source unique d'azote que lorsqu'ils sont en mélanges binaires.

Cette atténuation de leur toxicité est cependant incomplète car en leur présence les cultures sont toujours d'un type aberrant («masses nécrosées centrale» ou «basale» recouvertes d'un mince tapis de prothalles vivants). En aucun cas leur développement n'est normal comme il l'est sur le milieu-témoin (milieu de Knop à azote NO_3^-) (cultures entièrement vivantes, sans parties nécrosées).

Références

1. Sossountzov, I.: Action du glycocolle sur le développement in vitro des colonies prothalliennes de Gymnogramme calomelanos, Filicinée Polypodiacée. I. Morphologie générale et accroissement pondéral des colonies. — Physiol. Plant. 6: 723. 1953.
2. — Action de quelques acides aminés aliphatiques sur le développement in vitro des colonies prothalliennes de Gymnogramme calomelanos, Filicinée Polypodiacée. — Ibid. 7: 383. 1954.
3. — Etude de la croissance in vitro des prothalles de *Gymnogramme calomelanos* sur des milieux à concentrations variables en glycocolle et en alanine. — C. R. Soc. Biol. 144: 240. 1950.

Studies of the Enzymatic Degradation of Cellulose

By

Birgitta Norkrans

Institute of Physiological Botany

and

Bengt G. Rånby [1]

Institute of Physical Chemistry
University of Uppsala, Uppsala, Sweden
(Received December 31, 1955)

During the last years several studies of the enzymatic breakdown of cellulose have paid particular attention to the problem whether the enzyme attack proceeds from the end-group of the molecule or by means of a random splitting along the anhydroglucose chain in regions of equal accessibility Data have accumulated supporting the latter conception of an enzyme action analogo s to that of the α-amylase. The rapid loss of tensile strength of cellophane strips, and of viscosity of carboxymethylcellulose, the rapid depolymeriza ion of regenerated cellulose without a corresponding increase in the formation of reducing substances (Fåhræus 1946, Holden and Tracey 1950, Levinson and Reese 1950, Norkrans 1950 a), the attack on cellulose at about the same rate and differing only as to the degree of polymerization (Greathouse 1950, Walseth 1952), the independent formation of cellobiose and gl cose by using pure cellulase (Whitaker 1953), all these facts seem to indicate a random chain breaking.

Recently, however, Gilligan and Reese (1954) have published data about a fungal cellulase which seems to show this mode of action as well as an end-wise attack with cellobiose units as a result. Other investigators (Clayson 1943, Kitts *et al.* 1954) conclude that the decomposition proceeds only from the end-group of the molecule. (For further references, see Fåhræus 1956).

[1] Present address: Research Dept., American Viscose Corp., Marcus Hook, Pa. U.S.A.

The course of enzyme action might vary with the source of cellulolytic enzymes. Our earlier studies in the mechanism were performed with Tricholoma preparations (Norkrans 1950 a, b). It thus seemed interesting to extend the investigation to deal with enzymes from some other Hymenomycetes. Some data about Streptomyces enzymes have also been collected.

Material and Methods

The preparations used were from pure cultures of *Streptomyces sp* QM B 814 (cf. Reese, Gilligan, and Norkrans 1952) and from four different Hymenomycetes, viz. *Polyporus annosus* Fr. (cf. Norkrans 1950 a) and three strains of *Collybia velutipes* (Curt. ex Fr.) Quél, i.e. two monocaryotic wildtypes L 1 and L 11, as well as the dicaryotic combination (F 29/157 × F 28/42) between two arginineless mutants (cf. Aschan and Norkrans 1953).

The cultures were grown in 300 ml Erlenmeyer flasks on 0.5 per cent filter paper cellulose in mineral salts solutions supplemented with 0.25 g of yeast extract per litre (cf. Norkrans 1950 a). The development of the cellulolytic activity in the culture solutions was followed turbidimetrically during the incubation periods. All strains and species were incubated for time periods giving the maximum cellulolytic enzymatic activity. The time of incubation thus varied with the species studied: for Streptomyces sp and Polyporus annosus 14 days, Collybia velutipes L 1 12 days, L 11 15 days and (F 29/157 × F 28/42) 18 days. The mycelium was removed by centrifuging and filtration through a sintered glass filter "1G3", (through "3G5" in the case of Streptomyces). The filtrate was treated with 3 volumes of cold acetone for about 16 hours at about $+4°$ C. The precipitate was removed, dried in a vacuum desiccator until free from acetone-smell, suspended in water amounting to a twentieth of the filtrate volume, and shaken for one hour. About 65 per cent of the precipitate is water-soluble. The suspension was centrifuged and the supernatant solution preserved with 0.01 per cent merthiolate. Determinations by means of the micro-Kjeldahl method at different times on equivalent preparations gave the following total-nitrogen-values per ml solution: for Streptomyces of about 0.2 mg, Polyporus annosus 2.0 mg, Collybia velutipes: L 1 0.8, L 11 0.7 and (F 29/157 × F 28/42) 0.4 mg. About 90 per cent of the nitrogen could be dialysed.

A cellulose sol reprecipitated from a chemical grade wood cellulose solution containing 8 mg cellulose (DP 338) per ml was used as enzyme substrate. (For details concerning the sol preparation and methods of determining enzyme activity, see Norkrans 1950 b.)

The enzyme activity against cellulose sol has been measured turbidimetrically, the method being referred to as the photometric method in earlier papers (Norkrans 1950 a, b). The activity has also been expressed in terms of reducing substances, glucose formed, and changes in average degree of polymerization (DP). Furthermore the enzyme attack has been studied by electron microscopy of the cellulose sol.

In the turbidimetrical determinations the extinction values have been expressed according to the relation $Z = (e_0 - e_v) \times 10^3$ where $(e_0 - e_v)$ is the difference between the initial extinction value e_0 and that measured during the experiment (e_v). The
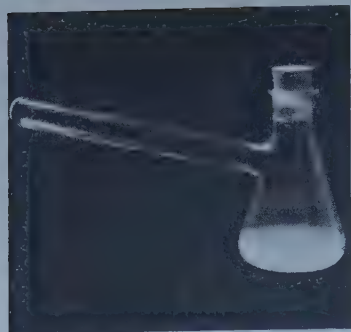
During
measurement.

During incubation.

Figure 1. *The side tube flask* (about $^1/_3$ of the natural size).

conditions were selected to give an initial extinction (e_0) of 1 and the extinction values plotted as the thousandfold. The various e-values have been calculated from the formula $\log \frac{I_0}{I}$.

The assay media for the turbidimetrical determinations were composed per flask of 20 ml cellulose sol, 10 ml enzyme preparation and 5 ml $^1/_4$ molar phosphate buffer, which would produce the optimum pH for the cellulolytic activity, viz. 6.8 for the Streptomyces preparation and 5.2 for all the other four. Furthermore 0.01 per cent merthiolate was added. These assay media were incubated in a special type of 125 ml Erlenmeyer flasks equipped with a side tube just below the flask neck (see Figure 1. The flask-type was described by Ryan and Schneider 1949, later on modified by B. v. Hofsten) During the turbidimetrical measurements the mixture — or part of it — was poured into the side tube. Such a tubeflask makes it possible to follow the turbidimetrical readings on one and the same mixture, of which there is sufficient to permit sampling for frequent tests for reducing substances, 2 ml per same and 2 samples each time. All flasks running in duplicates were shaken at 30° C and measured at intervals of several hours.

The amounts of reducing substances formed as breakdown products were deter mined according to Willstätter and Schudel and calculated as glucose.

The amounts of glucose formed were determined manometrically by using glucose oxidase (Keilin and Hartree 1945). The authors are greatly indebted to Dr. Elwyn T. Reese of the Pioneering Research Laboratories U.S. Army Quartermaster Corps, Natick, Mass., for supplying a highly purified oxidase. The changes in DP of precipitated cellulose under the influence of enzyme preparations of Polyporus annosus, Collybia velutipes L 1, and (F 29/157 × F 28/42) were determined after three different periods of varying hours. The constituents of the assay media were in the same proportions as in the corresponding turbidimetrical experiments, the amounts, however, were doubled. Three 200 ml Erlenmeyer flasks were set up for each enzyme preparation, one flask per time period. In addition to these series, the experiment comprised controls, one where the enzyme preparation was replaced by phosphate

buffer, and one where it was replaced by the corresponding enzyme preparation inactivated by heat-treatment (autoclaving to 2 atm.) sometimes called "HT"-enzymes.

Some paper chromatograms were run for analysis of the breakdown products in the filtrates. Descending flow was used at room temperature for 72 hours. Solv.nt: butanol, formic acid, and water (4 : 1 : 5). Developer: aniline hydrogen phthalate (Partridge 1949). Paper: Whatman n:o 4.

The degree of polymerization (DP) of the cellulose substrate during the enzymatic degradation was measured viscometrically and osmotically on the nitrated cellulose samples (for details of the nitration technique, cf. Dymling, Giertz, and Rånby 1955) The cellulose sol v as separated from the reaction solution by centrifugation, washed with 50 ml portions of distilled water three times to remove water soluble impurities (adsorbed enzymes, salts, etc). To avoid formation of hard lumps ("Verhornung"), the water was removed by successive washings with absolute alcohol and benzene and then air dried The resulting cellulose powder was further dried in the tubes in a vacuum desiccator over solid sodium hydroxide. As the amounts of samples were very small (0.04—0 08 g) all the following operations were also performed in the same centrifuge tubes (capacity ~ 60 ml). About 5 ml of nitration acid mixture ($HNO_3 : H_3PO_4 : P_2O_5$ in the proportions $48 : 50 : 2$) precooled to $-16°$ C was added to each tube and then vigorously stirred for dispersion of the sample. The tubes were closed with rubber stoppers and then brought into an icebath (0° C) during the nitration period (4 hours). The nitration was stopped by adding about 20 ml of ice and water under vigorous stirring and a rapid centrifugation to recover the cellulose nitrate formed. The nitrate was washed repeatedly with distilled water under centrifugation, stabilized by heating it with water for two periods of one hour in an oven at 105° C and washed again with distilled water at room temperature. The cellulose nitrate was air-dried and then dried over solid sodium hydroxide in a vacuum desiccator. No nitrogen analyses were made on these samples because of the small amounts of cellulose nitrates available The most likely nitrogen content is 13.6 per cent as analyzed for comparable samples.

The cellulose nitrate samples were transferred to dry Erlenmeyer flasks, weighed and dissolved in butyl acetate to concentration (c) of 0.1—0.2 g/100 ml. These solutions at the initial concentrations and after dilution were used for viscometric and osmotic measurements.

The viscometric measurements were performed with a capillary viscometer of the Ubbelohde type with the outflow time (t_0) for pure butyl acetate 149 sec. The dilution of the original solutions was made using pipettes directly in the viscometer and the outflow times (t) measured after careful mixing. The relative viscosity (η_{rel}) of a solution is defined as $\eta_{rel} = t/t_0$ and the specific viscosity as $\eta_{sp} = \eta_{rel} - 1$. The quantity log ($\eta_{sp}/c$) is plotted in diagrams against the concentration (c) and extrapolated to zero concentration giving the intrinsic viscosity $[\eta]$.

The osmotic measurements were made using capillary glass osmometers (Zimm and Myerson 1946) with very tight membranes (Ultracella-filters "Allerfeinst" No. 100, from Membran AG., Göttingen, Germany). The osmotic pressures were measured as height differences (Δh) in cm butyl acetate between the menisci in the "inner" and the reference capillary using a cathetometer with an accuracy in the readings of 0.002 cm. The results were plotted in diagrams as $\Delta h/c$ vs. and extrapolated to zero concentration giving $(\Delta h/c)_0$.

The molecular weights (M_n) were then calculated from the relation

$$M_n = \frac{RT}{d \, (\Delta h/c)_0}$$
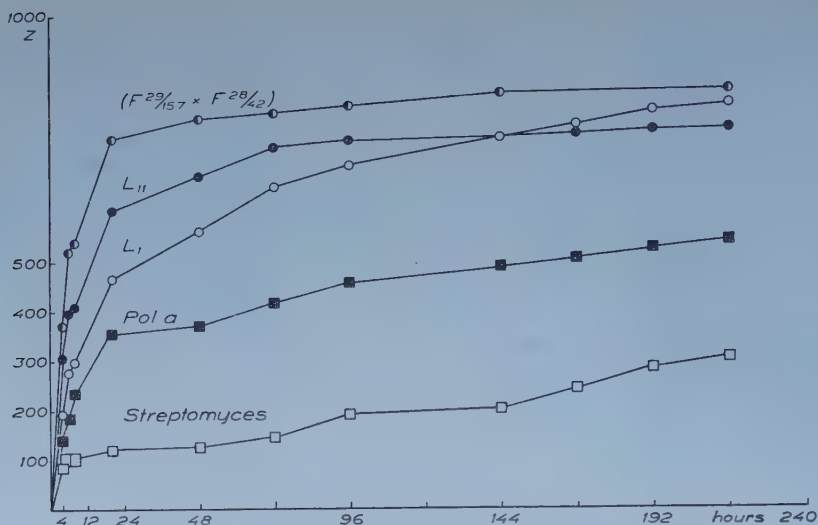
Figure 2. *Cellulolytic activity of some different enzyme preparations measured turbidimetrically.*

where R is the gas constant and T the temperature in absolute degrees (in this case $298°.1$ K, corresponds to $+25°$ C). Butyl acetate has a density (d) of 0.877 at $+25°$ C, and with Δh in cm and c in g/100 ml the gas constant (R) has the numerical value 848. With a nitrogen content of 13.6 per cent the molecular weight of the nitrated glucose unit is 288, which means that the degree of polymerization $(\overline{DP_n})$ of the cellulose nitrate is obtained by dividing the $\overline{M_n}$-values with 288.

Small amounts of the purified cellulose sols were also investigated by electron microscopy to study the effect of the enzyme attack (for a description of the method, cf. Rånby 1952). The sols were diluted with distilled water, irradiated with ultrasonic waves (22 kilocycles, about 1 watt/sqcm) for dispersion, prepared on membranes of Zapon lacquer (collodium), and shadowed by evaporation of metal (an alloy of gold-manganese in the weight proportion 1 : 1) in a high vacuum ($\sim 10^{-5}$ mm Hg) under inclined angle to increase the contrast. The electron microscopic exposures were made with a Siemens electron microscope (Type 1939, ÜM 100) at a magnification of 8 500 diameters (± 2 %). All micrographs reproduced in this paper are magnified optically four times to a total magnification of 34 000 $\times$.

Results and Discussion

The enzyme activity measured turbidimetrically. Determined turbidimetrically the enzyme preparations of the four different Hymenomycetes had a similar effect on the reaction course in the assay media (see Figure 2 and Table 1). During the first hours of incubation the Z-values increased rapidly,

Table 1. *Cellulolytic activity of some different enzyme preparations determined after an incubation time of varying hours expressed in Z-values, amounts of mg reducing substances, and glucose formed as well as values of average degree of polymerization (DP).*

	Phosphate buffer	Heat-treated enzyme (HT)	Untreated enzyme			
Collybia velutipes (F 29/157 × F 28/42)						
Incub. time in hours	216	216	4	20	96	216
Z			373	749	810	840
mg Red. subst.	0.9	1.2	3.9	6.3	6.5	6.7
mg Glucose	0	0.06 (0.4[1])	2.40	3.70	5.30	
$\overline{DP}_n$	338[2]	155[2]	54[2]	49[2]	49[2]	
Collybia velutipes L 1						
Incub. time in hours	216	216	4	48	168	168 + 48
Z			193	560	777	807
mg Red. subst.	0.9		3.0	3.7	4.1	6.2[3]
mg Glucose	0	0	1.80		2.90	3.65[4]
$\overline{DP}_n$	338[2]	230	61		51[2]	50
Collybia velutipes L 11						
Incub. time in hours	216	216	4	48	216	
Z			306	672	782	
mg Red. subst.	0.9		3.7	5.4	5.9	
Polyporus annosus						
Incub. time in hours	216	216	6	48	216	
Z	0.9		189	369	534	
mg Red. subst.	0.9		2.8	4.5	6.4	
mg Glucose	0	0	1.55	3.05	3.35	
$\overline{DP}_n$	338[2]	222	63	61	53	
Streptomyces sp.						
Incub. time in hours	216	216		20	216	
Z				128	302	
mg Red. subst.	0.9			1.3	1.7	

[1] After β-glucosidase treatment.

[2] Calculated from viscosity data; all other $\overline{DP}_n$-values are measured osmotically.

[3] The value obtained by this determination has been corrected by adding an amount of reducing substances approximatively corresponding to that removed at the enzyme substitution.

[4] The value obtained by this determination has been corrected by adding an amount of glucose corresponding to that removed at the enzyme substitution.

after the first twenty hours representing 60 to 90 per cent of the end-Z-values, which were determined after a reaction time of 216 hours. In this test as in all others, too, the preparation of *Collybia velutipes* (F 29/157 × F 28/42) showed the greatest total activity as well as the highest reaction rate. In the
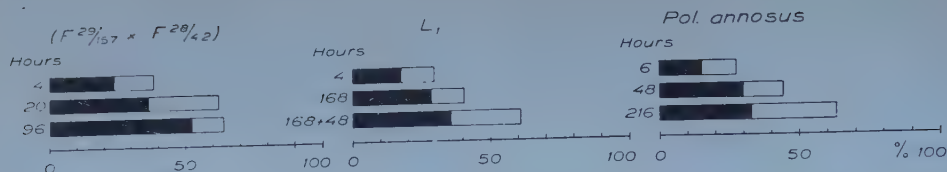
Figure 3. *Cellulolytic activity of some different enzyme preparations expressed as reducing substances* ☐ *and glucose* ■ *formed, calculated as a percentage of the initial cellulosic material.*

L 1-series a volume of fresh enzyme preparation was substituted for the initial one after 168 hours (marked "168+48 hours" in Table 1 and Figure 3). The fresh enzyme had a surprisingly small effect measured as turbidimetric Z-values.

The enzyme activity expressed in terms of reducing substances. The amounts of reducing substances formed in the assay media were calculated as glucose. Since the glucose determination with glucose oxidase (see Table 1 and Figure 3) as well as the paper chromatogram (Figure 4) indicate the presence of other reducing substances beside glucose among the breakdown products, this practise is of course, not quite proper. Anyhow, stated in this approximative way the breakdown did not in any case proceed to more than about 65 per cent of the total initial amount of cellulose (i.e. 9.14 mg cellulose per sample corresponding to 10.15 mg glucose). After an incubation time of 48 hours, the Pol. annosus and L 1 enzyme preparations produced only a very small if any increase in the amount of reducing substances. The increase in the L 1-series after 168 hours was certainly due to the addition of the fresh enzyme preparation. In spite of this addition the "65 per cent limit" was not exceeded. Probably, this limit is related rather to the morpho logical structure of the cellulose sol particles than to the enzyme inactivation. It is also possible that the small effect of the fresh enzyme is related to blocking of the active sites.

The enzyme activity expressed in terms of glucose formed. Table 1 and Figure 3 give an idea of the amount of glucose actually formed and the course of the glucose formation. Glucose did not in any determination correspond to the entire amount of reducing substances. The (F 29/157 × F 28/42) preparation, however, showed the highest glucose formation giving about 80 per cent of the total reducing substances as glucose, which means that about 50 per cent of the initial amount of cellulose was converted to glucose after an incubation time of 96 hours. In the case of the L 1- and Pol. annosus preparations the rate of glucose formation decreased earlier than the production of other reducing substances. These facts are tentatively interpreted to mean a greater sensitivity to the inhibitory effect of the breakdown products in the
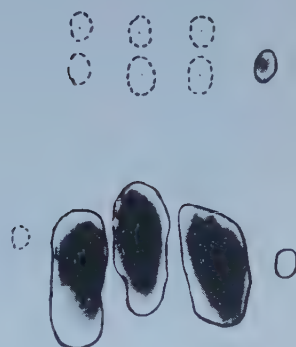
Figure 4. *Breakdown products of a cellulose sol after an incubation with an untreated Collybia velutipes (F 29/157× F 28/42) preparation for 4, 20, and 96 hours, respectively, and with a heat-treated one ("HT-enzyme") for 96 hours, called control.* Spots marked with dotted lines were visible only in UV-light.

glucose formation reaction than in the overall production of reducing substances.

The breakdown products studied by paper chromatography. The paper chromatogram (Figure 4) indicates the presence of cellobiose as well as another, slower-moving substance (R_f glucose $= 0.45$), presumably cellotriose, as reducing breakdown products besides glucose.

In most investigations the only products that have been found are cellobiose and glucose, but no intermediate dextrins. This fact has been used as an argument against the random cleavage theory. Be this as it may, oligosaccharides have in some cases been demonstrated (Jermyn 1952) and tentatively identified as cellotetraose (Hash and King 1954) and as cellotriose (Enebo 1954).

It has been urged, however, that oligosaccharides could always be secondary products, formed by a recombination of primary breakdown products by transglucosidase activity, assumed to be present in the enzyme preparations used. It is impossible to meet such an objection until a completely pure enzyme with only β-1,4-polyanhydroglucosidase activity has been obtained. At the present stage, studies of the cellulose residues therefore seemed most promising.

The enzyme activity studied by changes in the average degree of polymerization (DP). The measurements of the degree of polymerization (DP) of the cellulose substrates have given interesting views of the enzymatic attack on the cellulose chains.
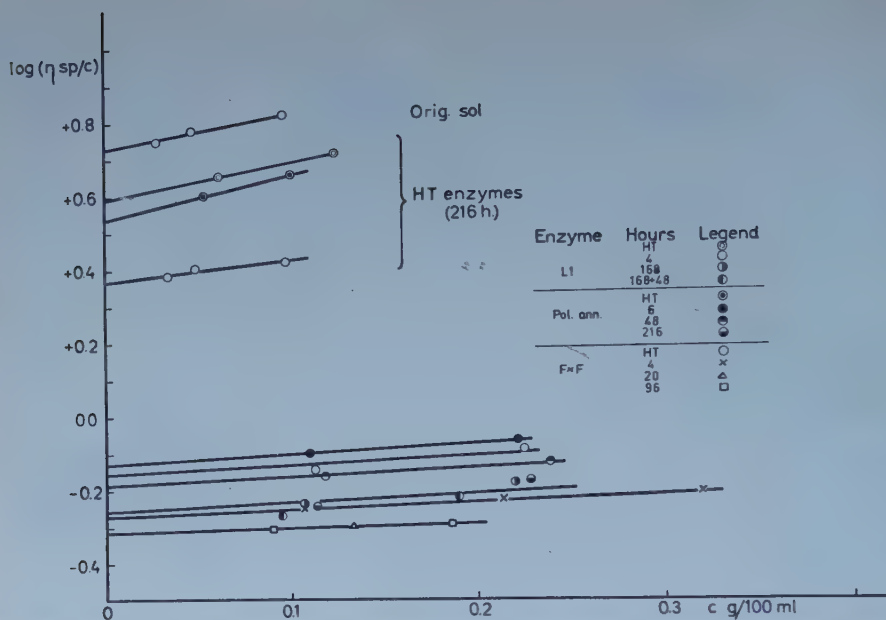
Figure 5. *Viscosity measurements in butyl acetate solution of cellulose sols after nitration.* "Orig. sol" is treated with buffer only, "HT enzymes" refer to experiments with heat-treated enzymes, and the other measurements refer to preparations with full activity. The extrapolated $[\eta]$-values for c=0 are given in Table 2 as $\overline{DP}_w = 100 \times [\eta]$.

Cellulose samples are generally polydisperse or polymolecular, i.e. they contain chain molecules of different length, and their molecular weight or degree of polymerization is given as an average defined in a certain way. Osmotic measurements give number averages molecular weights $(\overline{M}_n)$, i.e. the amount of substance is divided by the total number of molecules. In this case viscosity measurements give weight averages $(\overline{M}_w)$, i.e. the molecules are included in the average according to their weight (large molecules have then a stronger influence on the average than small molecules).[1] With these definitions it is clear that $\overline{M}_w > \overline{M}_n$ for all polymolecular substances, and that the quotient $\overline{M}_w/\overline{M}_n$ can be used to measure the degree of polymolecularity. For a random distribution of the molecular weights $\overline{M}_w/\overline{M}_n = 2$ according to calculations and measurements (cf. Gralén 1944, Jullander 1945 for reviews). This discussion is of course also valid for the average degrees of polymerization $(\overline{DP}_n$ and $\overline{DP}_w)$.

[1] The two averages are defined as:

$$\overline{M}_n = \Sigma M_i n_i / \Sigma n_i$$
$$\overline{M}_w = \Sigma M_i^2 n_i / \Sigma M_i n_i$$

where n_i means the number of molecules of the weight M_i.
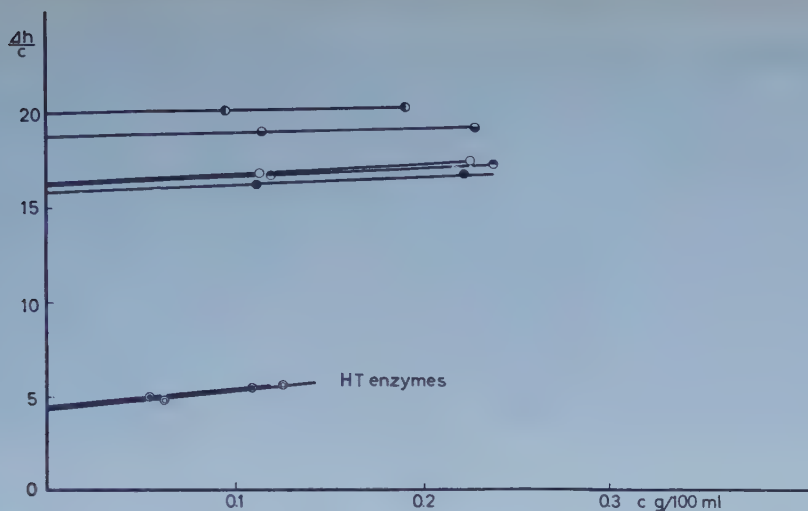
Figure 6. *Osmotic measurements on some of the samples included in Figure 5* (the same legends are used). Δh is in cm butyl acetate, extrapolated $(\Delta h/c)_0$-values are given in Table 2.

The viscosity measurements have been presented as log (η_{sp}/c) *vs.* c-diagrams in Figure 5 and the osmotic measurements as $\Delta h/c$ *vs.* c-diagrams in Figure 6. The cellulose of the original sol had an intrinsic viscosity $[\eta]=5.35$. With the most reliable K_m-constant (10×10^{-3}) — $[\eta]=K_m \cdot \overline{DP}_w$ — for cellulose nitrate (13.6 % N) in butyl acetate (cf. Immergut, Rånby, and Mark 1953) the cellulose has a $\overline{DP}_w=535$. The results of the osmotic measurements have been collected in Table 2. Because of the small amounts of material available no osmotic measurements were performed after treatment with the

Table 2. *Osmotic and Viscometric Measurements of Degrees of Polymerization.*

Enzyme	Hours	$(\Delta h/c)_0$	$\overline{DP}_n$	$\overline{DP}_w = 100 \cdot [\eta]$	$\overline{DP}_w/\overline{DP}_n$
Col. vel. L 1	HT	4.35	230	394	1.71
	4	.16.40	61	70	1.15
	168	—	$(51)^2$	55	$(1.09)^1$
	168 + 48	20.10	50	54	1.08
Pol. ann.	HT	4.50	222	347	1.56
	6	15.85	63	74	1.18
	48	16.30	61	65	1.07
	216	18.85	53	55	1.04
Col. vel. (F 29/157 $\times$ F 28/42)	HT	—	$(155)^2$	232	$(1.50)^1$
	4	—	$(51)^2$	54	$(1.06)^1$
	20	—	$(49)^2$	49	$(1.00)^1$
	96	—	$(49)^2$	49	$(1.00)^1$

[1] Quotients adopted from comparison with the *Col. vel.* L 1 and *Pol. ann.* series.
[2] $\overline{DP}_n$-values calculated using the adopted $\overline{DP}_w/\overline{DP}_n$-quotients.

(F 29/157×F 28/42)-enzymes. By comparison with the other series of samples, however, $\overline{DP}_n$-values were estimated for these cellulose samples, too. Using the same method, $\overline{DP}_n$ for the cellulose of the original sol was estimated to be 338.

The discussion below will be based on the number average $\overline{DP}_n$-values (cf. Figure 5, and Table 1 and 2). The initial enzymatic depolymerization proceeds very rapidly, representing a drop in $\overline{DP}_n$ from 338 to 51 during the first four hours under the influence of the (F 29/157×F 28/42) preparation, and to 61 and 63 in the L 1 and Pol. annosus assay media after four and six hours incubation, respectively. After that, the decrease proceeds gradually until cessation at a $\overline{DP}_n$ of about 50.

A comparison between the $\overline{DP}_n$-values and the corresponding glucose-values lend support to the random-splitting theory, most strikingly, perhaps, when one regards the values in the control series with heat-treated (F 29/157× F 28/42) preparation. The inactivation was not complete, but a certain activity was demonstrated in all the test methods used — the great heat-stability of cellulolytic enzymes had already been stated by Ziese (1931) and observed later on (Norkrans 1950 b). — Thus, there was an increase of 0.3 mg in reducing substances (comparisons made between controls with phosphate buffer and heat-treated enzyme), in glucose of 0.06 mg; in the paper chromatogram a spot could be detected by UV-light, and finally, the $\overline{DP}_n$ dropped from 338 to 155. In an enzyme attack from the end-group this drop should imply successive chain-breakings to more than 50 per cent along the cellulose chain. A corresponding amount of glucose or cellobiose or both could not be demonstrated. Analogous effects are found also with the two other enzyme preparations.

As has been said, the depolymerization seemed to proceed until a $\overline{DP}_n$-limit of about 50. Electron micrographs of such cellulose residues revealed a mass of distinct and fairly homogeneous particles about 300 Å long and about half as wide. These stood in clear contrast to those of the untreated cellulose sols, containing larger aggregates of longer disordered fibers (comp. Figures 7— 13) forming an irregular network or unresolved lumps. The enzymatic degradation apparently occurs in easily accessible regions of the cellulose aggregates leaving better crystallized and therefore more resistant particles as a residue. The rate of the enzymatic depolymerization is known to be highly dependent upon the crystallinity of the cellulose substrate (Norkrans 1950 a). The average length of the residual particles is in good agreement with the length of the cellulose chains in the enzyme-treated cellulose calculated from the measured DP_n-values (the length of a glucose unit in the cellulose chain is 5.15 Å) indicating that the particles observed on the micrographs are bundles of short cellulose chains. The electron microscopic in-
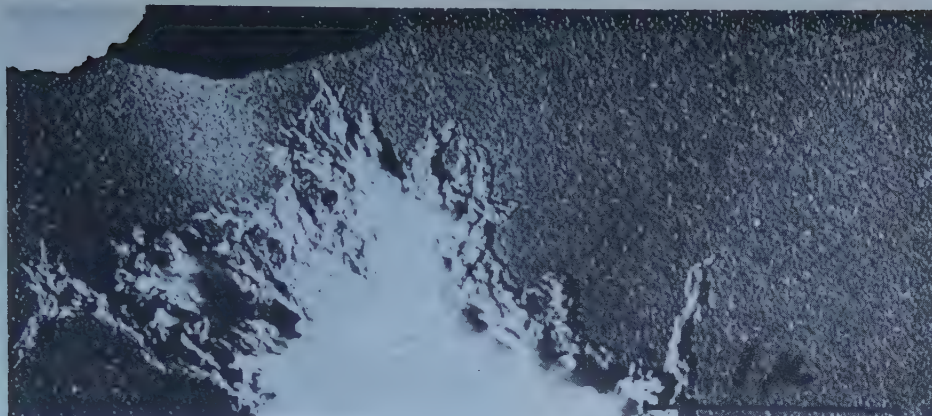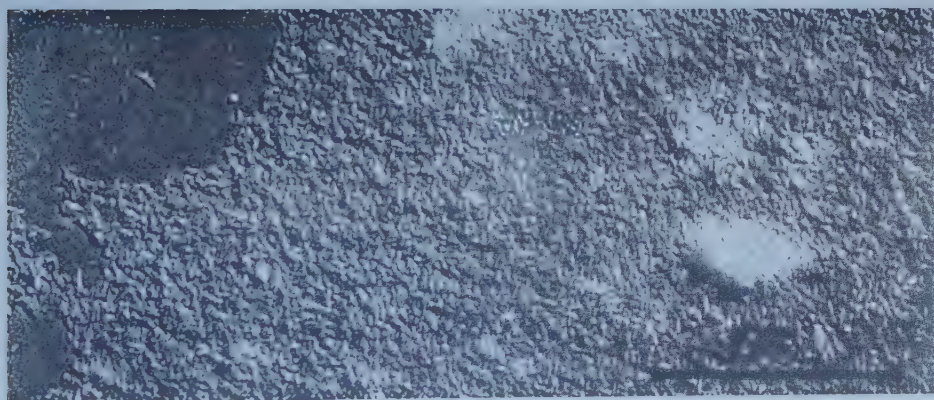
Figure 7.

Figure 8.

Figure 9.

Figures 7, 8, and 9. *Electron micrographs of cellulose sols from the Collybia velutipes L 1 series,* heat-treated enzyme Figure 7. 4 hours fully active enzyme Figure 8, and 168 +48 hours with active enzyme Figure 9. Magnification 34000 × (1 μ =corresponds to 34 mm on
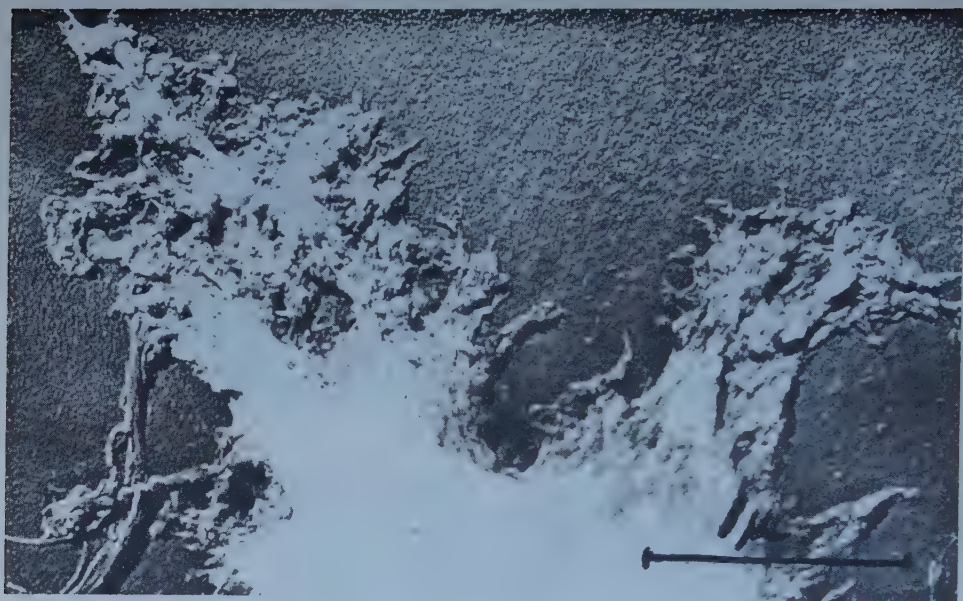
Figure 10.
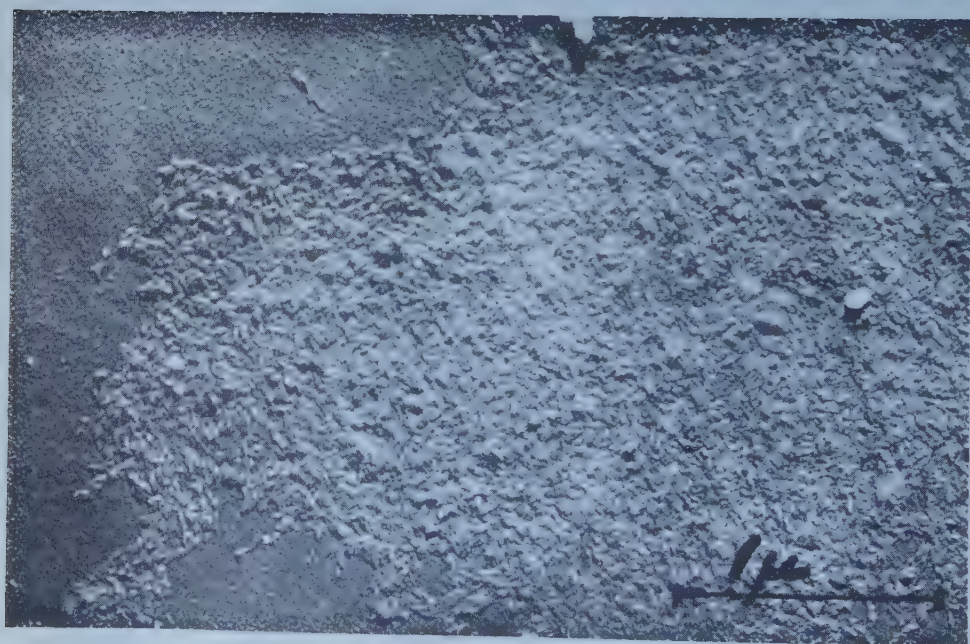
Figure 11.

Figures 10 and 11. *Electron micrographs of cellulose sols from the Polyporus annosus-series,* heat-treated enzyme (Figure 10) and 216 hours with fully active enzyme (Figure 11).
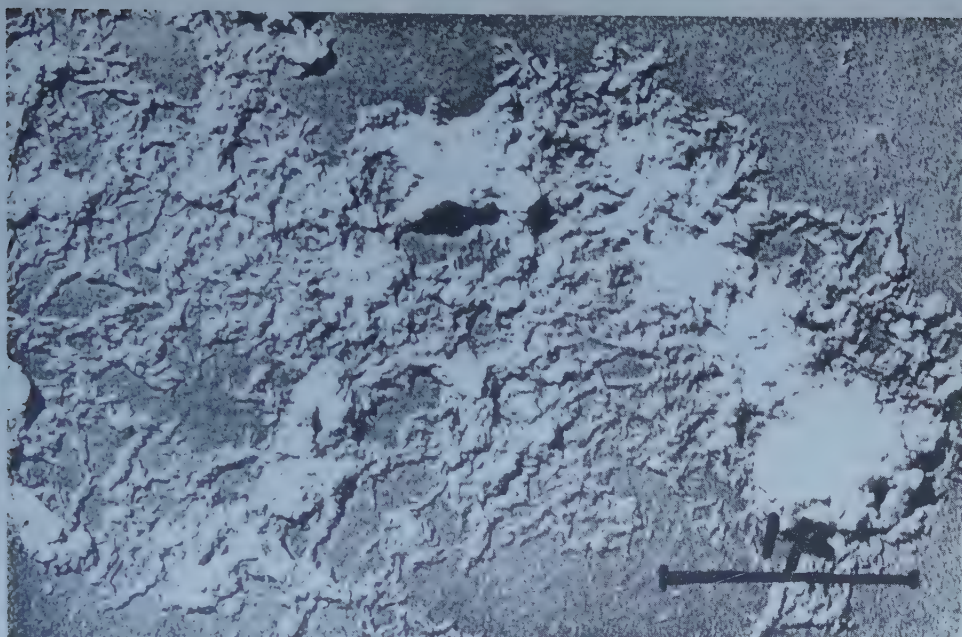
Figure 12.

Figure 13.

Figures 12 and 13. *Electron micrographs of cellulose sols from the Collybia velutipes* (F 29/157×F 28/42) *-series,* heat-treated enzyme (Figure 12) and 96 hours treatment with fully active enzyme (Figure 13).

vestigation also supports the random-splitting theory. Also the effect of degradation caused by heat-treated enzymes is indicated on the electron micrographs (comp. Figures 7, 10, and 12). The heat-treated (F 29/157 × F 28/42) enzyme is the most active and it also causes improved dispersion (comp. Figure 5 and Table 2).

The trends in the $\overline{DP}_w/\overline{DP}_n$-values of the two measured series (Table 2) indicate a gradual decrease in the polymolecularity as the depolymerization proceeds. An analogous effect has been observed in heterogenous acid hydrolysis of native wood and cotton cellulose fibers (Immergut and Rånby 1956). The absolute values of the $\overline{DP}_w/\overline{DP}_n$-quotients, however, are probably too low, because of the uncertainty in the K_m-constant used and because of some diffusion of low molecular weight cellulose through the osmotic membranes which is likely to occur and increase the measured $\overline{DP}_n$-averages.

It is interesting to compare the degradation caused by enzymes and that caused by acid hydrolysis. Boiling dilute mineral acid, e.g. 2.5-N sulphuric acid is definitely a stronger agent than the enzymes used in this investigation. A regenerated cellulose is depolymerized to a level of about 20 by acid hydrolysis (cf. Rånby 1951), a native wood cellulose (chemical grade pulp) to about 100 and a mercerized cellulose to about 50. The native and the mercerized fibers are degraded slowly and mainly on surfaces and certain cleavage planes by enzymes — an effect which probably is due to an assumed bulkiness of the enzyme protein molecules. Acid hydrolysis is catalyzed by H^+-ions of small size and very high mobility and penetration. A direct comparison of enzyme degradation and acid hydrolysis of regenerated cellulose sols as those used in this investigation, could give more information on the mechanism of the enzymatic attack on the cellulose chains.

Summary

1. The enzyme preparations tested for cellulolytic activity were from pure cultures of *Streptomyces* sp. QM B 814 and of four different Hymenomycetes, viz. *Polyporus annosus* Fr. and three strains of *Collybia velutipes* (Curt. ex Fr.) Quél, i.e. two monocaryotic wildtypes L 1 and L 11, as well as the dicaryotic combination (F 29/157 × F 28/42) between two arginineless mutants.

2. The enzyme activity against cellulose sol has been measured turbidimetrically, the method being referred to as the photometric method in earlier papers. (Norkrans 1950 a, b). The activity has also been expressed in terms of reducing substances, glucose formed, and changes in average degree of polymerization (DP). Furthermore the enzyme attack has been studied by electron microscopy of the cellulose sol.

3. In all tests the preparation of Collybia velutipes (F 29/157 × F 28/42) showed the greatest total activity as well as the highest reaction rate.

4. According to the turbidimetrical readings, as to the $\overline{DP}_n$-determinations, the enzyme preparations had a great influence on the cellulose sol during the first hours of incubation. The Z-values increased rapidly, after the first twenty hours representing 60 to 90 per cent of the end-Z-values. The initial $\overline{DP}_n$-value of 338 decreased to 50—60 in four (or six) hours.

5. The paper chromatogram indicates the presence of cellobiose as well as another, slower-moving substance, presumably cellotriose, as breakdown products besides glucose.

6. A comparison between the $\overline{DP}_n$-values and the corresponding glucose values lend support to the theory of a random-splitting of the cellulose chain.

7. Expressed in terms of reducing substances, the breakdown did not in any case proceed to more than about 65 per cent of the total initial amount of cellulose. The polymerization seemed to proceed until a $\overline{DP}_n$ of about 50. These limits seem to be related rather to the morphological structure of the cellulose sol particles than to the enzyme inactivation. Electron micrographs of such cellulose residues lend support to this conclusion, since they revealed a mass of distinct and fairly homogeneous particles about 300 Å long and about half as wide. These stood in clear contrast to those of the untreated cellulose sols, containing larger aggregates of longer disordered fibers forming an irregular network or unresolved lumps.

References

1. Aschan, K. & Norkrans, B.: A study in the cellulolytic variation for wildtypes and mutants of Collybia velutipes. I. — Physiol. Plant. 6: 564. 1953.
2. Clayson, D. H. F.: The enzymic degradation of cell wall substances. Part. II. — Chemistry and Industry 62: 49. 1943.
3. Dymling, E., Giertz, H. W. & Rånby, B. G.: Fractional dissolution of nitrated carbohydrates in native fibers. — Svensk Papperstidn. 58: 10. 1955.
4. Enebo, L.: Studies in cellulose decomposition by an anaerobic thermophilic bacterium and two associated noncellulolytic species. — Diss. Stockholm 1954.
5. Fåhræus, G.: Enzyme preparations from cellulose-decomposing bacteria. — Experientia 2: 413. 1946.
6. — Celluloseaufbauende und celluloseabbauende Fermente. — Handbuch der Pflanzenphysiologie. VI. Springer Verlag. 1956. In print.
7. Gilligan, W. & Reese, E. T.: Evidence for multiple components in microbial cellulases. — Canad. J. Microbiol. 1: 90. 1954.

8. Gralén, N.: Sedimentation and diffusion measurements on cellulose and cellulose derivatives. — Diss. Uppsala. 1944.

9. Greathouse, G. A.: Microbiological degradation of cellulose. — Textile Res. J. 20: 227. 1950.

10. Hash, J. H. & King, K. W.: Demonstration of an oligosaccharide intermediate in the enzymatic hydrolysis of cellulose. — Science 120: 1033. 1954.

11. Holden, M. & Tracey, M. V.: A study of enzymes that can break down tobacco-leaf components. 2. Digestive juice of Helix on defined substrates. — Biochem. J. 47: 407. 1950.

12. Immergut, E. H., Rånby, B. G. & Mark, H. T.: Recent work on molecular weight of cellulose. — Ind. Eng. Chem. 45: 2483. 1953.

13. Immergut, E. H. & Rånby, B. G.: Heterogeneous acid hydrolysis of native cellulose fibers. — Ib. 1956. In print.

14. Jermyn, M. A.: Fungal cellulases. I. General properties of unpurified enzyme preparations from Aspergillus oryzae. — Austral. J. Sci. Res. B. Biol. Sci. 5: 409. 1952.

15. Jullander, I.: Studies on nitrocellulose. — Arkiv Kemi, Mineral. Geol. 21 A, No 8. (1945). — Diss. Uppsala, 1945.

16. Keilin, D. & Hartree, E. F.: The use of glucose oxidase (notatin) for the determination of glucose in biological material and for the study of glucose-producing systems by manometric methods. — Biochem. J. 42: 230. 1948.

17. Kitts, W. D. & Underkofler, L. A.: Digestion by rumen microorganisms. Hydrolytic products of cellulose and the cellulolytic enzymes. — J. Agric. Food. Chem. 2: 639. 1954.

18 Levinson, H. S. & Reese, E. T.: Enzymatic hydrolysis of soluble cellulose derivatives as measured by changes in viscosity. — J. Gen. Physiol. 33: 601. 1950.

19. Norkrans, B.: Influence of cellulolytic enzymes from Hymenomycetes on cellulose preparations of different crystallinity. — Physiol. Plant. 3: 75. 1950 a.

20. — Studies in growth and cellulolytic enzymes of Tricholoma. — Symb. Bot. Ups. XI. 1: 1. 1950 b.

21. Partridge, S. M.: Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. — Nature 164: 443. 1949.

22. Rånby, B. G.: The collodial properties of cellulose micelles. — Faraday Soc. Disc. No. 11: 158. 1951.

23. — The physical characteristics of alpha-, beta-, and gammacellulose. — Svensk Papperstidning 55: 115. 1952.

24. Reese, E. T., Gilligan, W. & Norkrans, B.: Effect of cellobiose on the enzymatic hydrolysis of cellulose and its derivatives. — Physiol. Plant. 5: 379. 1952.

25. Ryan, F. J. & Schneider, L. K.: The consequences of mutation during the growth of biochemical mutants of Escherichia coli. III. The inhibition of histidine-independent bacteria by histidineless bacteria in aerated cultures. — J. Bact. 58: 191. 1949.

26. Walseth, C. S.: The influence of the fine structure of cellulose on the action of cellulases. — TAPPI 35: 233. 1952.

27. Whitaker, D. R.: Purification of Myrothecium verrucaria cellulase. — Arch. Biochem. and Biophys. 43: 253. 1953.

28. Ziese, W.: Ueber die Einwirkung von Fermenten des Magensaftes von Helix pomatia und solcher des Gerstenmalzes auf Celluloseglykoläther. — Zeitschr. physiol. chem. 203: 87. 1931.

29. Zimm, B. H. & Myerson, I.: A convenient small osmometer. — J. Am. Chem. Soc. 68: 911. 1946.

Starch Formation in Ripening Pea Seeds

By

Carl Erik Danielson

Aktiebolaget Findus, Bjuv, Sweden
(Received January 21, 1956)

One of the most important questions in the canning and quick freezing of peas is the determination of the proper time of harvest. It is generally known that unripe peas contain sugar which at the end of the ripening process is converted into starch (1). The formation of starch in the peas is undesirable because the quality of the canned product decreases with increasing starch content of the peas. It is, however, important that the peas are not harvested too early because then the yield is low. Many investigations have been carried out in order to develop suitable routine methods for the determination of the degree of ripening of peas. Some of these methods are based on chemical or physical-chemical properties. Kertesz (2, 3) determined alcohol-soluble substances in the seeds and from these determinations it was possible to get a measure of maturity. Nielsen (10) determined the maturity in peas by determination of the starch content, and Lee (4, 5) determined the specific weight of the seeds. Some methods are based on the fact that unripe peas are more tender than ripe peas. Thus, ripe peas give a higher resistance than unripe peas when they are sheared between two grids, or penetrated by a series of steel pins. This fact has been used by Martin, Lueck and Sallee (8) in the construction of the tenderometer, and by Lynch and Mitchell (6, 7) in the maturometer. The tenderometer, which apparatus is used universally in pea canneries, gives a "tenderometer reading" varying from 70 for very unripe seeds to about 170, for overripe seeds. In order to be able to refer the results described in this paper to practical canning, some determinations of maturity have been made using the commercial tenderometer.

The methods described above for the determination of maturity have been

developed in order to get a practical and rapid routine method for industrial work. The experiments described in the present paper have been carried out in order to get a more clear picture of what happens when the sugar is converted into starch during the ripening process.

1. Experimental

In the experiments, which were carried out in 1954, seeds of peas, *Pisum sativum*, Profusion variety, were used. The pods were harvested in the morning and transported directly to the laboratory, where the seeds were separated from the hulls and analyzed.

In order to get a homogeneous seed material, the pods were first classified according to the stage of development. The pea plant forms flowers acropetally, *i.e.* the lowest pods are developed earlier than those pods which grow near the top of the plant. The lowest pods belong to Node 1 and the higher pods which are developed later belong to Node 2, Node 3 etc. In the experiments described here three nodes were used.

The fresh weight of the seeds was determined by weighing 100 seeds. Moisture was determined after drying at 105° C for 24 hours. For the determination of starch and reducing sugar, 10 grams of seeds were ground in 40 ml. ethanol for 2 minutes, in a small Waring Blendor. The suspension was transferred to a 250 ml. beaker, which was placed at 70 C for 60 minutes; by this procedure the starch was precipitated. The ethanol solution was filtered and the ethanol was evaporated by heating on a waterbath. During the evaporation water was added. Proteins were precipitated with lead acetate and saturated Na_2HPO_4-solution. The filtered solution was analyzed for total sugar after hydrolysis with HCl, using Fehling's solution and iodometric titration. The starch precipitate was dried at 70 C and ground with quartz sand in a mortar. The precipitate was transferred to a acetate buffer of pH 4.5 and heated for 30 minutes in boiling water to cause swelling. The starch was hydrolyzed with taka-diastase at 38° C over night. The disaccarides formed were determined quantitatively with the same method as described above for the determination of sugar in the ethanol extract. The results of the determinations are shown in Table 1.

2. The sugar content of ripening peas

Usually the starch and sugar contents are expressed as per cent of the fresh weight or dry substance of the seeds (1). The present author, however, considers that, in kinetic investigations on soluble substances all calculations should be based on actual concentrations, *i.e.* the sugar content in the seeds should be expressed as per cent of the total amount of water in the seeds.

Table 1. *Fresh weight, dry substance, sugar and starch in ripening peas, Profusion variety.*

Date of harvest	Fresh weight g./100 seeds			Dry substance % of fresh weight			Sugar % of fresh weight			Starch % of fresh weight			Tenderometer reading		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
21.7.54	5.7	5.2	2.9	15.8	15.2	15.3	3.2	2.4	2.6	2.9	3.1	3.0			
	12.9	7.2	3.1	15.8	15.2	15.6	4.6	3.0	2.1	2.9	2.9	2.8			
23	14.0	6.1	2.5	15.9	15.3	16.3	4.8	3.8	—	3.1	3.1	3.1			
	18.3	10.2	3.6	16.0	15.5	15.6	—	4.2	2.5	3.4	3.7	3.4			
25	25.6	17.1	9.3	17.1	16.2	16.0	5.8	3.6	3.7	3.0	3.0	2.8			
27	32.4	23.3	14.5	18.4	17.2	16.2	6.1	6.1	4.7	2.7	2.9	3.4			
29	37.3	36.0	17.5	18.8	17.9	16.7	5.7	6.6	6.1	2.7	2.6	2.4	76		
2.8.54	44.4	38.8	24.4	19.3	19.8	19.2	6.1	6.5	5.4	2.8	2.6	3.0			
4	61.5	43.6	33.9	21.5	20.5	18.5	6.4	6.3	6.1	2.8	2.8	2.0	89		
6	61.6	47.1	37.0	19.1	20.9	19.6	6.2	6.8	6.8	3.4	3.4	2.6	100	91	
8	73.8	54.0	46.4	23.0	20.8	19.6	6.1	6.7	6.3	4.0	3.2	2.8	115	103	88
10	66.4	71.0	59.2	22.5	22.6	20.7	6.0	6.0	6.8	3.7	3.4	2.8	119	108	100
12	75.8	72.2	68.6	22.4	20.9	22.1	5.6	6.7	6.8	5.3	5.1	4.7			
	69.6	79.8	76.8	23.3	23.3	21.1	4.0	5.0	6.0	—	—	—	140	124	108
16	86.6	70.0	76.6	24.8	22.7	22.2	2.9	3.4	4.2	6.4	6.1	5.1	168	154	127
18	76.4	75.4	69.2	25.5	24.4	23.0	2.3	3.1	4.2	4.0	2.8	3.1			
20	82.8	93.8	83.1	25.7	23.5	23.7	1.7	2.8	2.3	6.7	6.4	6.0			

I, II, III refer to the different nodes.

This method of calculation is of special importance when the volume of the system under investigation increases. The correctness of this assumption is shown in Figure 1, where the sugar content (g./100 seeds) is plotted against the water content (g./100 seeds) of pea seeds in different stages of ripening. Figure 1 shows that, in the first stage of the ripening process, the sugar content of the seeds is a linear function of their water content. At a water content of 56 g. H_2O/100 seeds, the sugar concentration decreases rapidly. From Figure 1, it is clear that the sugar concentration, calculated on the water content of the seeds, is constant over practically the whole ripening process. The sugar concentration is the same for the seeds in the different nodes. An interesting fact is that if the straight line in Figure 1 is extrapolated to a sugar content=0, the water content 3.5 g. H_2O/100 seeds is obtained, *i.e.* 100 not too small seeds contain 3.5 g. water, which is apparently free from sugar. Most of this water is probably found in the cell walls and in the cytoplasm.

The determinations presented in Table 1 and Figure 1 were also carried out on peas of the Lincoln variety. Exactly the same results were obtained. All experiments were repeated in 1955 with the same results.

As a comparison, Figure 2 show the experimental results from 1925 of Bisson and Jones (1) on peas of the Dwarf Telephone variety. If the sugar content is expressed as per cent of the water content of the seeds, the results of Bisson and Jones confirm in detail the results of the present author.
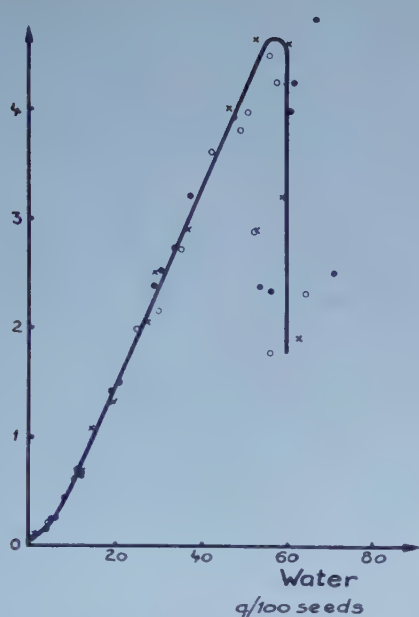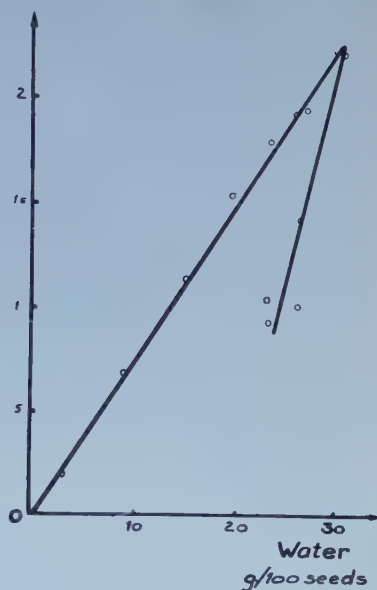
Figure 1. *Sugar content as a function of water content in ripening pea seeds, Profusion variety.* ○ = Node 1, ● = Node 2, × = Node 3.

Figure 2. *Sugar content as a function of water content in ripening pea seeds, Dwarf Telephone variety.* The experimental values were obtained from Bisson and Jones (1).

3. The starch content in ripening peas

The starch which is formed in the ripening peas is probably, in the main part, insoluble in water. Thus, it is not quite correct to refer starch concentration to the water content of the seeds in the same way as for the sugar content. However, this method has been used as a comparison in Figure 3. It can be seen that the formation of starch occurs in two stages. In order to get a more clear picture of the relationship between the sugar and starch contents in ripening peas, the logarithm of the concentrations of sugar and starch in the water phase have been plotted against time in Figure 4. In the calculation of the sugar concentration from the values in Table 1, a correction has been made. The "apparently sugar-free water", 3.5 g H_2O/100 seeds, has been subtracted from the experimentally determined water content of the seeds. Only the values from the determinations on seeds from Node 1 have been used.
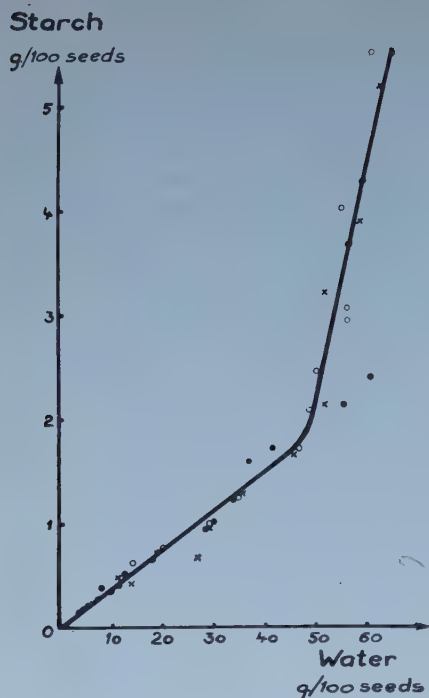
Figure 3. *Starch content as a function of water content in ripening pea seeds, Profusion variety.*

Discussion

Figure 4 shows most clearly the changes in carbohydrate content in ripening peas. The concentration of total sugar in the water phase is constant during a long period of the ripening process. The water phase in the seeds of Node 1 contains about 8.2 per cent reducing sugar. The same value was obtained for peas of the Lincoln variety, and if the author's method of calculation is used, the experimental values of Bisson and Jones on Dwarf Telephone give the same sugar concentration in the water phase of the seeds (1). The sugar in peas consists of 95 per cent sucrose (1). It is very difficult to understand, how the sugar concentration can be maintained constant at a high value in the water phase during the ripening process. Here further studies are necessary, especially on the concentrations of the sugar solutions which are transported from the leaves to the developing seeds. The hypothesis that the accumulation of sugar in the seeds is regulated by the osmotic conditions in the cell sap is very tempting but the problem is probably much more complicated.

At a certain stage of the ripening process (August 11 in Figure 4) the concentration of sugar decreases rapidly. This happens when the growth is
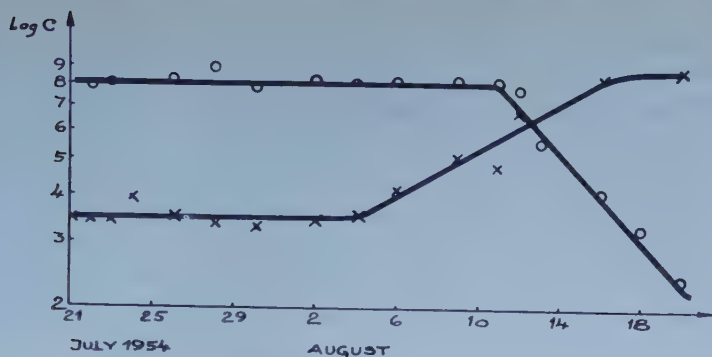
Figure 4. *The logarithm of the concentrations of sugar* (○) *and starch* (×) *in the water phase of pea seeds, Profusion variety, as a function of time.*

nearly completed. The reaction is a first order reaction. In order to get an answer to this sudden drop in sugar concentration a comparison with the curve for the starch concentration must be made.

From Figure 4, it can be seen that the starch concentration, based on the water content of the seeds, is constant during the first part of the ripening process, but that from August 4, the starch concentration increases rapidly according to a first order reaction. If the two curves in Figure 4 are compared it can be clearly seen that the sugar is converted into starch.

The problem is, however, more complicated than could be assumed from Figure 4. It is not so simple that the uptake of sugar stops on August 11 and the remaining sugar in the seeds is quantitatively converted into starch. Table 1 shows that the dry weight of the seeds increases by about 43 per cent after August 11, which fact means that carbohydrates are transported to the seeds even during the last stage of the ripening process. Part of the sugar which is transported to the seeds is also used for the synthesis of substances other than starch, *e.g.* cellulose, and some sugar is used for respiration. An interesting fact is that the rapid starch synthesis begins August 4, but the sugar concentration does not decreases until 7 days later. The same tendency can be seen from the experimental values obtained by Bisson and Jones (1). Thus it seems probable that the synthesis of starch is not regulated by the amount of accumulated sugar in the cells, but by other factors, *e.g.* the activity of the enzyme systems which synthesize the starch. The author has worked with the following hypothesis.

During the whole ripening process sugar is accumulated in the seeds at such a rate that a constant concentration of about 8 per cent is maintained in the water phase. At the same time, starch is synthesized at such a rate. that the concentration of starch in the expanding system is constant. At a certain stage (August 4) the rate of starch formation is increased, and the

reaction is of first order until August 16, at which date the peas are regarded as ripe. During the first week of the intense starch formation, the sugar concentration is constant, but after August 11 the growth of the seeds and synthesis of starch are so intense that the sugar concentration decreases, *i.e.* sugar cannot be transported to the seeds at the same rate as sugar is used up in the seeds. A steady state is, however, maintained, *i.e.* the sugar concentration decreases as a first order reaction.

The sudden increase in the velocity of starch formation (August 4, Figure 4) can be explained in different ways. One possibility which must be investigated is that not the same type of starch is synthesized during the whole ripening process. McCready *et. al.* (9) found that in wrinkled peas, the ratio amylose to amylopectin increases as the peas mature. Another possibility is that the starch-synthesizing enzymes are activated at this stage of ripening. The synthesis of starch and the synthesis of proteins both occur through high-energy phosphorous bonds. The synthesis of proteins is practically finished at August 4 (Danielson, unpublished). Thus there is a possibility that all phosphorous in the seeds is first used for the synthesis of proteins, and then for the starch synthesis.

Figure 4 also gives some views on the purely practical problems, which were mentioned in the introduction of this paper. The tenderometer readings for the peas under investigation were T=85 on August 3, T=117 on August 10 and T=168 on August 16. The starch curve in Figure 4 shows that the intense starch formation occured between August 3 and August 16, *i.e.* when the tenderometer readings changed through the whole range of measurement. Thus, the tenderometer reading is a relative measure of the starch content of the seeds. If the tenderometer reading is a linear function of the starch content, it should also, according to Figure 4, be a logarithmic function of time. This assumption only holds if the peas are grown in a constant climate, where the number of "accumulated heat units" (11) during the ripening period is a linear function of time. This was the case in 1954 when the experiments described here were carried out.

In the commercial canning of peas it has been found empirically that peas should be harvested when the tenderometer reading is about T=115, *i.e.* about August 11 in Figure 4. Thus the optimal harvest time for peas is just before the sugar concentration in the water phase of the seeds begins to decrease.

Summary

1. The sugar and starch contents have been determined at different stages of ripening, in peas of the Profusion variety.

2. Sugar is accumulated in the pea seeds at such a rate, that the concentration in the water phase is maintained at 8—9 per cent. At the end of the ripening process, the sugar concentration decreases according to a first order reaction.

3. Starch is synthesized with two different velocities. The hypotheses that two different kinds of starch are synthesized, or that the starch-synthesizing enzymes are activated, are presented.

4. For canning purposes, peas should be harvested just before the sugar concentration in the water phase of the peas begins to decrease.

References

1. Bisson, C. S. & Jones, H. A.: Changes accompanying fruit development in the garden pea. — Plant. Physiol. 7: 91, 1932.
2. Kerterz, Z. I.: New objective methods to determine maturity of canned peas. — Food Inds. 6: 168, 1934.
3. — The chemical determination of quality of canned green peas. — N.Y. St. Agric. Exp. Sta. Tech. Bull. No. 233, 1935.
4. Lee, F. A.: Determining maturity of frozen peas. Rapid objective method. — Ind. Eng. Chem., Anal. Ed. 13: 38, 1941.
5. — Objective methods of determining maturity of peas, with special reference to the frozen product. — N.Y. St. Agric. Exp. Sta. Tech. Bull. No. 256, 1941.
6. Lynch, L. J. & Mitchell, R. S.: Physical measurement of quality in canning peas. — Commonwealth Sci. Ind. Res. Organization, Bull. No. 254, Melbourne, 1950.
7. — — Optimal harvest time of pea canning crops. — Commonwealth Sci. Ind. Res. Organization, Bull. No. 273, Melbourne, 1953.
8. Martin, W. M., Lueck, R. H. & Sallee, E. D.: Practical application of the tenderometer in grading peas. — Canning Age 19: 146, 1938.
9. Mc Cready, R. M., Guggolz, J., Silviera, V. & Owens, H. S.: Determination of starch and amylose in vegetables. Application to peas. — Anal. Chem. 22: 1156, 1950.
10. Nielsen, J. P.: Rapid determination of starch. — Ind. Eng. Chem. Anal. Ed. 15: 176, 1943.
11. Seaton, H. L.: Scheduling plantings and predicting harvest maturities for processing vegetables. — Food Technol. 9: 202, 1955.

Glutamic Acid Metabolism in Green and Etiolated Barley Leaves [1]

By

Aubrey W. Naylor [2] and N. E. Tolbert

The Biology Division, Oak Ridge National Laboratory,
Oak Ridge, Tennessee
(Received January 30, 1956)

Glutamic acid plays a central role in the nitrogen metabolism of plants and animals. It quickly penetrates membranes of cells (Najjar and Gale, 1950), and is metabolized readily when supplied to bacteria and the tissues of plants or animals. Glutamic acid is known (1) to be utilized in the synthesis of proteins; (2) to be deaminated to α-ketoglutaric acid and metabolized by way of the Krebs cycle; (3) to serve as a precursor in the general metabolism of arginine and proline; (4) to be transformed into its amide, glutamine; and (5) to be decarboxylated, yielding γ-aminobutyric acid.

Much of the earlier work on amino acid metabolism in plants was reviewed by Chibnall (1939), and more recent findings on amino acid and protein metabolism in plants are described by Steward and Thompson (1954) and Webster (1955). There is also an excellent summary in *Amino Acid Metabolism* (McElroy and Glass, 1955).

Changes in glutamic acid metabolism are induced quickly by either growth-stimulating substances such as indoleacetic acid (Chibnall, 1954), or growth-inhibiting substances (Petersen and Naylor, 1953). Sudden withdrawal of oxygen in animals is accompanied by almost explosive changes in the amount

of glutamic acid present (Richter and Dawson, 1948; Takagaki, 1955). Relatively little is known about the speed of transformation of glutamic acid in plants.

In this investigation, the many normal metabolic products associated with glutamic acid utilization have been determined by sampling at intervals after the leaf is fed this amino acid as a uniformly C^{14}-labeled compound. Glutamic-C^{14} acid has been supplied under several environmental conditions, and a comparison has been made of metabolic products arising from it. Anaerobically in green leaves, and aerobically in etiolated leaves, γ-aminobutyric acid was formed rapidly as the major product from glutamic acid.

Materials and Methods

The leaves were kept throughout experimental treatment in small glass chambers provided with ports that permitted continuous circulation of any chosen gas mixture. When light was used, intensities of 600 ft.-c. or more were obtained at the leaf surface with a reflector photoflood bulb. Heat was minimized by passing the light through a water filter maintained at constant temperature (Withrow and Elstad, 1953) and by rapidly flowing air or nitrogen (Linde, spectrographically pure) through the glass vessel. These measures prevented the temperature from rising above 28° C.

Young Sacramento barley seedlings were always employed; these seedlings were 8—10 days old and had been grown in soil or nutrient culture. The terminal 5—7 cm. of the first leaf was severed with a sharp razor blade and the cut end submerged in an aqueous solution of glutamic-C^{14} to a depth of 1—2 mm. This solution was made by dissolving 1 mM of uniformly C^{14}-labeled glutamic acid (Nuclear Chicago) having a specific activity of 100 μc./mM in 2 ml. of water. By using cut-off ends of 2 ml. centrifuge tubes as vessels, a minimum quantity of labeled glutamic acid was required. In practice this amounted to 20 μl. of solution containing 1 μc. of C^{14}. This quantity was usually taken up within 15 to 30 minutes, after which time water was added.

At the end of the feeding period, which was usually one-half, one, two, and three hours, the leaves were killed by freezing in liquid nitrogen. The frozen leaves were reduced to a fine powder, covered with 30 per cent methanol-water, transferred to a centrifuge tube, and boiled for one minute in a water bath. After centrifugation, the supernatant was reduced to 0.1 ml. by incubating at no more than 60° C. and by blowing a stream of dry, filtered air over the surface.

An aliquot of the concentrate equivalent to one-tenth to one-fifth of the leaf was chromatographed on sheets of 18-×22-inch Whatman No. 1 paper with water-saturated phenol in the first direction, and n-butanol-propionic acid-water in the other (Benson et al., 1950). The chromatograms were exposed to No-Screen X-ray film for two weeks to one month to locate the labeled compounds. The amount of radioactivity contained by each compound was determined by counting the area directly on the paper with a Geiger-Müller tube.

Amino acids and organic acids were identified by their R_f values and, in addition, each radioactive compound cochromatographed identically with its specific non-

Fig. 1 A.

labeled compound that was added in sufficient quantity to be detected by spray tests. Organic acids were rechromatographed in ether-acetic acid water and detected by bromocresol green indicator spray (Denison and Phares, 1952). Amino acids were detected by ninhydrin spray, and generally sufficient quantities of amino acids were present on the original chromatograms to give a positive spray test. Sucrose was eluted with water. and part of the elute was rechromatographed with carrier sucrose; the remainder, after hydrolysis with Dowex-50 in the acid form for 30 minutes at 100° C., was cochromatographed with carrier glucose and fructose which were detected by specific sugar spray tests (Block et al., 1955).

γ-Aminobutyric acid was identified by its R_f values in several solvents (Lederer and Lederer, 1955). Its radioactivity cochromatographed with chemically synthesized γ-aminobutyric acid in all the solvents tested. In fact. it was present generally in the leaves in sufficient quantities to be detected by the ninhydrin spray on the original chromatograms without carrier. The compound was converted into its ureide by incubation with potassium thiocyanate in a steam bath for 48 hours. This ureide had the same R_f values as the authentic ureide of γ-aminobutyric acid and gave a
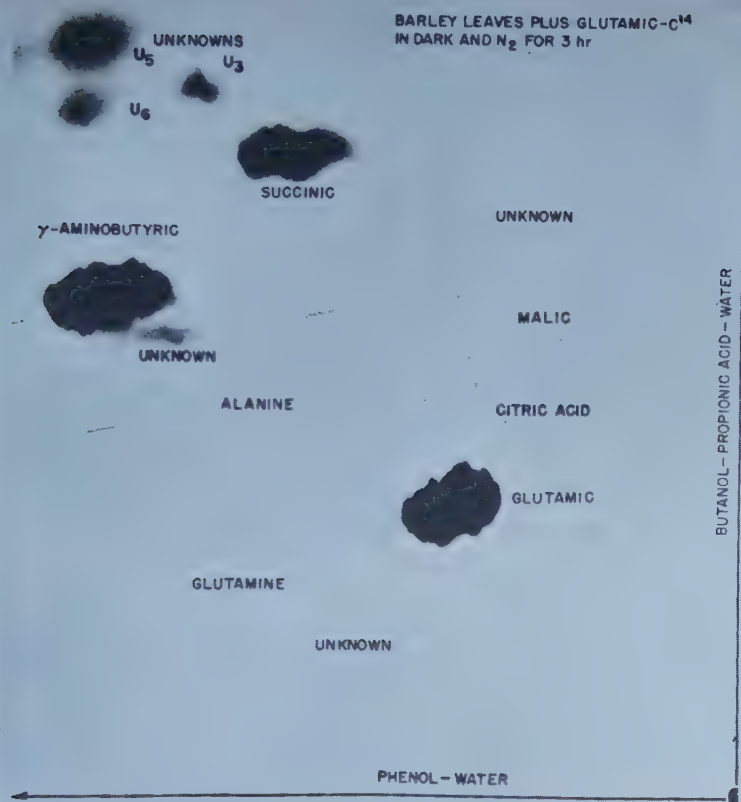
Fig. 1 B.

yellow color when tested with a p-dimethyaminobenzaldehyde spray (Block et al., 1955). The γ-aminobutyric acid did not complex with $CuCO_3$ impregnated on the paper chromatograms in the path of movement of the compounds during the first solvent development, whereas α-amino acids characteristically do so (Crumpler and Dent, 1949). Ethanolamine is the only known ninhydrin-positive nitrogen-containing compound which has nearly the same R_f value in the chromatographic solvents employed, and which would not be complexed by the $CuCO_3$. Radioactivity in the postulated γ-aminobutyric acid, however, did not cochromatograph with ethanolamine.

Results and Discussion

The major water and alcohol soluble compounds deriving a portion of their carbon skeletons from glutamic acid in relatively short intervals of time under aerobic and anaerobic conditions in the dark are depicted in the

Table 1. *Percentage* [1] *of* C^{14} *in soluble products from glutamic-*C^{14} *acid metabolism in green barley leaves.*

Products	Dark & Air 3 hrs.	Light & Air 1 hr.	Dark & Nitrogen 3 hrs.	Light & Nitrogen 1 hr.
Glutamic Acid	22.6	31.3	56.6	63.2
Glutamine	32.2	32.9	0.6	11.3
Aspartic Acid	6.5	2.3	trace	0.2
Alanine	2.1	2.9	trace	1.3
Threonine	0.6	0.3	trace	0.0
γ-Aminobutyric Acid	1.4	2.3	32.3	15.6
Succinic Acid	2.3	3.3	5.4	1.5
Malic Acid	24.6	11.3	0.5	0.7
Citric Acid	4.3	0.7	0.6	1.2
Glucose-Fructose	0.0	0.7	0.0	0.0
Sucrose	0.4	5.4	0.0	0.6
Unknowns No. 1, plus No. 2	2.1	1.1	0.9	0.3
Unknown No. 3	0.5	0.6	0.6	0.3
Unknown No. 4	0.0	1.3	trace	1.7
Unknown No. 5 plus No. 6..	0.0	0.0	2.5	1.6

[1] Differences between 100 per cent and the sum of the values in each column is caused by small amounts of other compounds not listed in this table.

autoradiographs shown in Figure 1 A—B. The absence of oxygen drastically altered glutamic acid metabolism. These differences for the major products have been expressed in quantitative terms in Table 1. The data are from representative experiments involving four different experimental conditions of light or dark and an atmosphere of air or nitrogen. Although the time interval during which glutamic acid was available was varied from one-half hour to three hours, only 3-hour experiments in the dark were required to yield extensive transformations of the glutamic acid, whereas one hour in the light best showed utilization of the glutamic acid with relatively little evidence of circulation of the C^{14} into the photosynthetic cycle.

The major product from glutamic acid under aerobic conditions was glutamine, the γ-amide of glutamic acid. Formation of glutamine did not occur under anaerobic conditions. This was expected, since its synthesis requires high-energy phosphate from oxidative phosphorylation (Elliott, 1951; Webster, 1953), or perhaps from photosynthesis. Under strictly anaerobic conditions, which can be obtained only in the dark, glutamic acid was decarboxylated next to the amino group, and yielded γ-aminobutyric acid as the major product. When light and nitrogen gas were supplied, the results were part way between those for leaves in nitrogen and those in sufficient oxygen, as in air. From this the inference may be drawn that as glycolysis occurred releasing some CO_2, photosynthesis proceeded. yielding small amounts of oxygen for a low rate of respiration.

There is an active glutamic acid decarboxylase present in barley (Beevers, 1951). A crude enzyme preparation made from crookneck summer squash according to Schales and Schales (1946), and a more highly purified one made according to Weinberger and Clendenning's (1952) method were used to prepare radioactive γ-aminobutyric acid. When glutamic-C^{14} acid was used as the substrate, it was quantitatively converted to γ-aminobutyric acid as determined by chromatographic and autoradiographic analysis. These isotope data thus confirm previous manometric work on the mode of action of glutamic decarboxylase. Presumptive evidence given in Table 1 indicates that this enzyme functions actively in green barley leaves under both aerobic and anaerobic conditions. From present data one cannot determine whether the rate of γ-aminobutric acid synthesis from glutamic acid was increased under anaerobic conditions, or whether this rate was normally rapid and further metabolism of the γ-aminobutyric acid was inhibited by anaerobiosis. If the latter is the case, γ-aminobutyric acid could occupy an important metabolic pathway from glutamic acid to succinic acid.

Labeling in malic acid indicates that anaerobically, the pathway of metabolism for glutamic acid through α-ketoglutaric acid into malic acid was not functioning rapidly — a result which is expected. Anaerobiosis, however, had less effect on the distribution of label in succinic and citric acids than is readily accountable. Thus there is the possibility of alternate and undescribed pathways of synthesis of these acids. In the relatively short-term experiments reported here, there seemed to be no more than a slight tendency for radioactivity from the glutamic carbon chain to appear in proline and intermediates in the ornithine cycle.

Distribution of the C^{14} label in other products identified on the chromatograms indicates that there was no rapid respiration of the glutamic acid carbon chain to CO_2 in the light and then fixation into photosynthetic products. These results are in agreement with the hypothesis that, in light, photosynthetically produced reducing power slows the rate of respiration in products associated with the tricarboxylic acid cycle by maintaining the coenzymes involved in a comparatively reduced state (Calvin, 1949; Calvin and Massini, 1952) Table 1 shows, however, that glutamic acid was metabolized approximately three times as fast in light as in the dark. Metabolic products in light and dark were about the same. Rates of synthesis associated with the tricarboxylic acid cycle seemingly are not slowed, but, in fact, are increased by light. Bidwell et al. (1955) also have found evidence that the tricarboxylic acid cycle is in operation in the light.

Several apparently important compounds from glutamic acid metabolism remain unidentified. As indicated by Figure 1 A –B, all of these had large R_f values. Possibly some of these are cyclic or aromatic in nature. Partially

Table 2. *Percentage of C^{14} in soluble products derived from glutamic-C^{14} acid metabolized in etiolated barley.*[1] (Calculated on the basis of amount of glutamic acid remaining at the end of each time interval.)

Time (hrs.)	Glutamine	γ-Aminobutyric	Succinic	Malic	Aspartic	Alanine	Unidentified compounds
1/2	24	29	5	9	24	—	9
1	18	10	4	5	30	3	30
1-1/2	15	4	6	10	21	—	43
2	16	6	11	8	31	3	26
2-1/2	24	2	5	13	39	3	14
3	26	2	4	7	23	3	33

[1] Gas phase was air; light intensity was 600 ft. c.

Differences between 100 per cent and the sum of the values in each line is caused by small amounts of compounds not listed in the table.

because yields of these compounds have been small, they are not yet identified. Compounds U_1 and U_2 had R_f values approximately that of γ-aminobutyric acid. U_3 and U_4 had R_f values approximately that of lactic and fumaric acids respectively, but they did not cochromatograph with these acids. Compound U_4 is characterized by being produced in the light, but not in the dark. Unidentified U_5 and U_6 appeared in quantity only when the leaves were in a nitrogen atomosphere. Possibly these are anaerobic products from glutamic acid, or perhaps they are derived from the large amounts of γ-aminobutyric acid present.

Time course studies were conducted to obtain a qualitative expression of the relative activity of enzyme systems competing in the metabolism of glutamic acid. These experiments were run with eight-day-old etiolated seedlings in air and light. Most rapid uptake of glutamic acid occurs in the light, and because of no photosynthetic $C^{14}O_2$ fixation, etiolated seedlings can be used for several hours without confusion in interpretation. Tolbert and Gailey (1955) have shown that etiolated Thatcher wheat begins photosynthesis only after 4—5 hours in the light, although a "dark" type of CO_2 fixation is present from the beginning. Results of glutamic acid feedling to etiolated barley leaves are given in Table 2.

During the 3-hour feeding period, there was a continuous increase in the percentage of radioactive glutamic acid that was metabolized by the leaves. When calculated on the basis of the percentage of glutamic-C^{14} acid utilized up to a given time, the data indicate that equilibrium reactions are quickly established that yield approximately equivalent amounts of alanine for any given interval of time. Relatively small fluctuations were noted in glutamine, succinate, malate, and aspartate. The most striking data are those for γ-aminobutyric acid. At the end of the first half hour more of the C^{14} label

had gone into this 4-carbon compound than into glutamine (5 carbons). Thereafter there was a decline in the relative amount of label in γ-amino-butyric acid. The apparent tendency of γ-aminobutyric to level off at a relatively lower concentration after 2 ½ hours infers that is was reaching an equilibrium concentration after initial higher levels. It would thus seem that there is a time lag during which equilibrium reactions involving γ-amino-butyric acid are established.

Decarboxylation to γ-aminobutyric acid appears to be one of the most accessible of the several pathways by which glutamic acid may be metabolized in the young barley leaf. As the oxygen supply approaches a critical point, this reaction becomes the dominant one—approximately 80 per cent of the derived radioactivity was present in this compound after 3 hour's anaerobiosis. In etiolated leaves it was the first major product, even in air. Thus this amine may be suspected of playing an important role in metabolism.

γ-Aminobutyric acid is found in a great variety of plant tissues and is present in the brain. That it may contribute its nitrogen in the eventual formation of protein has been indicated by Thompson et al. (1953). Many investigations have failed to show whether γ-aminobutyric acid is a constituent of protein. An equivocal report of its presence in the protein hydrolyzate of orange juice has appeared (Wedding and Sinclair, 1954). Attempts in this laboratory to verify these findings with comparable hydrolyzates have yielded negative results.

An active γ-aminobutyric acid transaminase has been found in brain tissue (Bessman, Rossen, and Layne, 1953). Wilson, King, and Burris (1954) looked for the enzyme in lupine seedlings. but their results were inconclusive. γ-Aminobutyric acid transaminase has, however, been reported to be present in pea roots (Miettinen and Virtanen, 1953). Through transamination, succinic semialdehyde would be formed which in turn might be transformed to succinic acid.

Summary

Products from the metabolism of uniformly labeled glutamic-C^{14} acid by green and etiolated Sacramento barley leaves have been analyzed chromatographically after time intervals of ½ to 3 hours under four different experimental conditions of light or dark, with atmospheres of air or nitrogen.

1. The rate of glutamic acid metabolism was faster in light than in darkness.

2. Glutamine, malate, citrate, aspartate, and γ-aminobutyric acid were

associated with aerobic metabolism in the light. Therefore it appears that light did not interfere with the functioning of the Krebs cycle.

3. Glutamic acid was decarboxylated anaerobically *in vivo* to γ-amino-butyric acid; about 80 per cent of the metabolized amino acid appeared in this amine.

4. In etiolated seedlings in air and light, γ-aminobutyric acid appeared at first as rapidly, or more rapidly, from glutamic acid as did glutamine. After a short period during which γ-aminobutyric acid accumulated, it subsequently decreased to a small equilibrium amount by the time the etiolated leaf had been in the light 2 1/2 hours.

5. γ-Aminobutyric acid was the only product *in vitro* from the action of glutamic acid decarboxylase on glutamic-C^{14} acid as shown by paper chromatography and autoradiography.

6. *In vivo,* and especially anaerobically, the carbon label appeared in several compounds as yet unidentified. High R_f values of some of the compounds suggest ring configurations.

The authors are indebted to Miss Pat Kerr for her able technical assistance throughout the course of this study.

Some of the material in this paper was presented at the American Society of Plant Physiologists meetings in East Lansing, Michigan, in September, 1955.

References

Beevers, H.: An L-glutamic acid decarboxylase from barley. — Biochem. J. 48: 132. 1951.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W.: The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. — J. Am. Chem. Soc. 72: 1710. 1950.

Bessman, S. P., Rossen, J. & Layne, E. C.: Gamma-aminobutyric acid transamination in brain. — J. Biol. Chem. 201: 385. 1953.

Bidwell, R. G. S., Krotkov, G & Reed, G. B.: The influence of light and darkness on the metabolism of radioactive glucose and glutamine in wheat leaves. — Canad. J. Botany 33: 189. 1955.

Block, R. J., Durrum, E. L. & Zweig, G.: A Manual of Paper Chromatography and Paper Electrophoresis. — 2nd ed., Academic Press, Inc., 1955.

Calvin, M.: The path of carbon in photosynthesis. VI. — J. Chem. Educ. 26: 639. 1949.

Calvin, M. & Massini, P.: The path of carbon in photosynthesis. XX. The steady state. — Experientia 8: 445. 1952

Chibnall, A. C.: Protein Metabolism in the Plant. — Yale University Press, 1939.

— Protein metabolism in rooted runner bean leaves. — New Phytologist 53: 31. 1954.

Crumpler, H. R. & Dent, C. E. Distinctive test for α-amino acids in paper chromatography. — Nature 164: 441. 1949.

Denison, F. W., Jr. & Phares, E. F.. Rapid method for paper chromatography of organic acids. — Anal. Chem. 24: 1628. 1952.

Elliott, W. H.: Studies on the enzymic synthesis of glutamine. — Biochem. J. 49: 106. 1951.

Lederer, E. & Lederer, M.: Chromatography. Elsevier Publishing Co., 1955.

McElroy, W. D. & Glass, H. B., Eds.: Amino Acid Metabolism. The Johns Hopkins Press, 1955.

Miettinen, J. K. & Virtanen, A. I.: Nitrogen metabolism of pea and alder. Transamination of gamma-aminobutyric acid and I(+)-citrulline with alpha-ketoglutaric acid. — Acta chem. Scandinav. 7: 1243. 1953.

Najjar, V. A. & Gale, E. F.: The assimilation of amino-acids by bacteria. — Biochem. J. 46: 91. 1950.

Petersen, E. L. & Naylor, A. W.: Some metabolic changes in tobacco stem tips accompanying maleic hydrazide treatment and the appearance of frenching symptoms. — Physiol. Plant. 6: 816. 1953.

Richter, D. & Dawson, R. M. C.: The ammonia and glutamine content of the brain. — J. Biol. Chem. 176: 1199. 1948.

Schales, O. & Schales, Selma S.: Glutamic acid decarboxylase of higher plants. III. Enzymatic determination of L(+)-glutamic acid. — Arch. Biochem. 11: 445. 1946.

Steward, F. C. & Thompson, J. F. In "The Proteins", II A. pp. 513—594 H. Neurath and K. Bailey, Eds. Academic Press, 1949.

Takagaki, G.: Ammonia formation and glutamic acid in brain. — J. Biochem. (Japan) 42: 131. 1955.

Thompson, J. F., Pollard, J. K. & Steward, F. C.: Investigations of nitrogen compounds and nitrogen metabolism in plants. III. γ-Aminobutyric acid in plants, with special reference to the potato tuber and a new procedure for isolating amino acids other than α-amino acids. — Plant Physiol. 28: 401. 1953.

Tolbert, N. E. & Gailey, F. B.: Carbon dioxide fixation by etiolated plants after exposure to white light. — Plant Physiol. (In press).

Webster, G. C.: Enzymatic synthesis of glutamine in higher plants. — Plant Physiol. 28: 724. 1953.

— Nitrogen metabolism. — Ann. Rev. Plant Physiol. 6: 43. 1955.

Wedding, R. T. & Sinclair, W. B.: A quantitative paper-chromatographic study of the amino acid composition of proteins and juice of the orange. — Botan. Gaz. 116: 183. 1954.

Weinberger, P. & Clendenning, K. A.: Glutamic carboxylase of the mature wheat leaf. — Canad. J. Botany 30: 755. 1952.

Wilson, D. G., King, K. W. & Burris, R. H.: Transamination reactions in plants. — J. Biol. Chem. 208: 863. 1954.

Withrow, R. B. & Elstad, V.: Water-cooled lamp systems with refluxing aqueous filters. — Plant Physiol. 28: 334. 1953.

Interaction of Manganese and 2, 4-Dichlorophenol in the Enzymatic Destruction of Indoleacetic Acid

By

WILLIAM S. HILLMAN and ARTHUR W. GALSTON

Josiah Willard Gibbs Research Laboratories
Department of Plant Science
Yale University
New Haven, Connecticut, U.S.A.
(Received February 2, 1956)

Introduction

In recent years various workers have analyzed the mechanism of the enzymatic destruction of indoleacetic acid (IAA) by plant breis (2, 5, 7, 12, 13). It is now generally agreed that a peroxidative reaction is involved in the ultimate destruction of IAA (2, 7, 10) and that various monophenols enhance this reaction (4, 7). Considerable confusion exists, however, regarding the effect of Mn^{++} ion, which has been reported as enhancing (5, 13), inhibiting (1, 2) or being without effect (13) on the IAA oxidase systems of various plant breis. The data to be presented below suggest an explanation of these apparently discrepant results, based on a demonstrable interaction between the effects of Mn^{++} and 2,4-dichlorophenol (DCP) on the enzyme activity.

Materials and Methods

Seeds of *Pisum sativum,* variety Alaska, were obtained from Asgrow, Inc., of New Haven, Connecticut, sown in vermiculite (Mica-Gro Type B, supplied by Platt Seed Co., Branford, Connecticut) and allowed to develop in darkness to the 3-internode stage (3). In several experiments, the temperature was $13 \pm 1°$ C., and in others, $26 \pm 1°$ C. Most of the experiments were conducted with breis of 2nd internode tissue, but young internode tissue from the growing region was used in others.

Breis were prepared by quick-freezing 2.5 g. of tissue at $-16°$ C., grinding in a
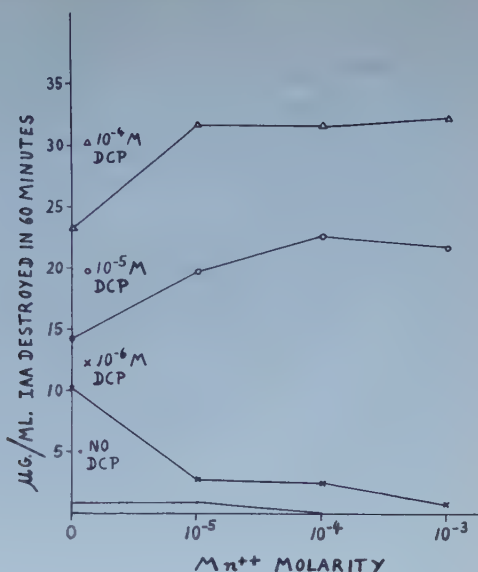
Figure 1. *IAA oxidase activity of etiolated pea internode brei as a function of Mn++ concentration, at various levels of DCP.*

chilled mortar with additions of $1/50$ M pH 6.1 KH_2-Na_2HPO_4 buffer, filtering through cellulose tissue to remove coarse debris, and making up to 25 ml. with buffer. Brei was stored at ca. 4° C. and used within an hour after preparation.

IAA-oxidase activity was assayed by determining residual IAA in aliquots removed from reaction mixtures incubated at 26° C. in an Aminco-Dubnoff metabolic shaking incubator. IAA was determined according to Tang and Bonner (11) except that readings were made after 20 minutes instead of 30 minutes. Each reaction mixture contained 1.0 ml. brei, 2.0 ml. pH 6.1 $1/10$ M phosphate buffer, the addenda if any, water, and IAA in a total volume of 10.0 ml. IAA solution to give an initial concentration of 35 µg/ml. $(2 \times 10^{-4}$ M) was added at zero time. IAA standards and a turbidity blank (reaction mixture without IAA) were used in each set of IAA determinations. No IAA destruction took place in any reaction mixture without brei. Incubation was in darkness except for brief exposures to light of less than 20 foot candles intensity during removal of aliquots.

Mn^{++} was supplied as $MnCl_2 \cdot 4H_2O$. IAA and 2,4-dichlorophenol (DCP) were obtained from the Eastman Chemical Company, Rochester, New York.

Results

Activity with no addenda was very low in all experiments, resulting in the destruction of 1.0 µg./ml. IAA per hour at most. Figure 1 summarizes the results of a typical experiment on the interaction of various levels of Mn^{++} and DCP. It is evident that the rate of IAA destruction increases with increasing DCP, as previously reported (4). The effect of Mn^{++}, however, appears to depend upon the level of DCP present, inhibiting IAA destruction
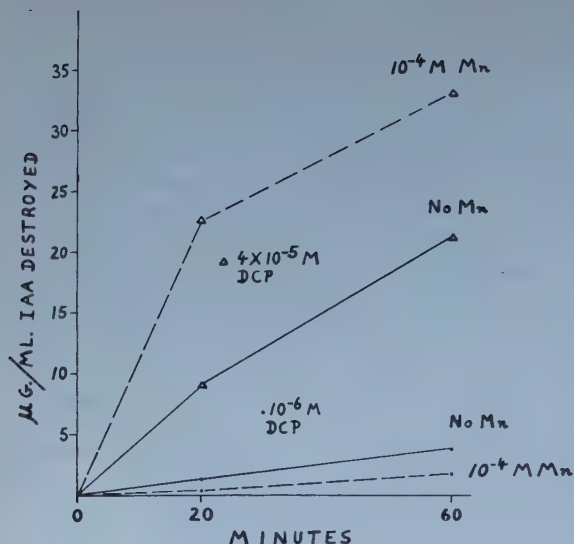
Figure 2. *Time course of IAA destruction at two levels of DCP with and without* 10^{-4} *M* Mn^{++}.

at low DCP concentrations and enhancing it at high DCP concentrations. In the absence of both DCP and Mn^{++} there is a small but significant destruction of IAA which is completely abolished by 10^{-4} and 10^{-3} M Mn^{++}. At a DCP concentration of 10^{-6} M, where IAA destruction is considerable, 10^{-4} M Mn^{++} inhibited IAA destruction more than 75 percent in the experiment graphed. At the higher levels of DCP, however, the addition of the same concentration of Mn^{++} results in a marked enhancement of activity. Figure 1 underestimates this enhancement, since at the high activity conferred by the higher DCP levels the progress curve is no longer linear by the end of 60 minutes. Figure 2, from the data of another experiment, illustrates the destruction of IAA as a function of time, and shows that 10^{-4} M Mn^{++} can cause an increase of more than 100 percent in the initial rate of IAA destruction in the presence of 4×10^{-5} M DCP. The same concentration of Mn^{++} again inhibits IAA destruction when only 10^{-6} M DCP is present.

Qualitatively similar results were obtained in all experiments, whether breis from young or mature tissues grown at $13°$ or $26°$ C. were used, although quantitative differences between experiments were often observed (cf. Figures 1, 2).

Discussion

The most probable explanation of the effect of DCP on IAA destruction is that it supplements or replaces a naturally-occurring phenolic cofactor (4). In terms of this explanation, our data suggest that the discrepant reports on

the action of Mn^{++} are due to the use of breis differing in their concentration of the endogenous phenol. Breis starting with a relatively high concentration would show enhancements by the same levels of Mn^{++} which would inhibit breis with less of the endogenous phenol. The data obtained do not suggest that the ratio of Mn^{++} to DCP *added* is the significant factor in activity. In Figure 1, e.g., 10^{-4} *M* Mn^{++} in the presence of 10^{-5} *M* DCP (10 : 1) enhanced activity, while 10^{-5} *M* Mn^{++} inhibited in the presence of 10^{-6} *M* DCP (also 10 : 1). It would be necessary to know the endogenous activity of the cofactor replaced by DCP, as well as that of Mn^{++} (or the factor replaced by Mn^{++}), in order to obtain an estimate of the optimal ratio, if any.

No adequate explanation for all the effects of Mn^{++} and of phenols such as DCP on reactions involving peroxide is at hand. There is evidence that Mn^{++} reduces phenol oxidation products (6), reduces peroxides directly (14) and increases peroxide formation *in vitro* (8) and *in vivo* (11), while the substituted monophenols have been implicated in oxidation-reduction systems (7), peroxidase activation (9) and *in vivo* peroxigenesis (11). The scheme suggested by Kenten and Mann (6) for the oxidation of certain dicarboxylic acids is attractive here. An intermediate phenol oxidation product produced by peroxidase action is reduced by Mn^{++}, and the final oxidation (in this case, that of IAA) is carried out by the oxidized manganese. In such a system, a great excess of Mn^{++} over phenol would inhibit IAA destruction by allowing reduction of the phenol oxidation product without a significant lowering of the ratio of reduced to oxidized manganese. With excess phenol, however, Mn^{++} would limit and added Mn^{++} would enhance destruction.

It should be noted that the present results were obtained with reaction mixtures in which the brei was highly diluted (less than 1 percent by fresh weight) and in which activity in the absence of added DCP was very low. In experiments with more concentrated reaction mixtures, or with breis from bud tissue, the rates with no addenda were higher, and additions of Mn^{++} either inhibited slightly or enhanced activity, suggesting less limitation by the phenol component. Even in such experiments, however, Mn^{++} in the presence of high (ca. 2×10^{-5} *M*) DCP concentrations always caused enhancements much greater than the added effects of either DCP or Mn^{++} alone.

Goldacre et al (4) observed that the activity of well-dialyzed pea epicotyl brei was enhanced by Mn^{++} while that of crude brei was inhibited. They suggested that the inhibition might be due to Mn^{++} competition with IAA in reducing a phenolic oxidation product, but admitted that the Mn^{++} enhancement of the activity of well-dialyzed brei was difficult to explain on this basis. This is particularly true since DCP enhancement of the activity of this brei was greater than its enhancement of crude brei, suggesting that

the phenol was more limiting in dialyzed brei. Our present data suggest that the dialyzed brei used by Goldacre et al, may have lost not only phenolic cofactor but manganese also, so that Mn^{++} was limiting after dialysis, although present in supraoptimal amounts before it.

The more than fourfold enhancement of IAA destruction in pineapple stem preparations by 5×10^{-3} M Mn^{++} reported by Gortner and Kent (5) appears to be the highest Mn^{++} effect in the literature, and probably reflects a very high level of endogenous phenolic cofactor. The fact that added DCP had little or no effect on the enzyme supports this conjecture.

Summary

IAA destruction by breis from internode tissue of etiolated peas was inhibited by 10^{-5} to 10^{-3} M Mn^{++} ion at low concentrations (10^{-6} M) of DCP, or in its absence. The same Mn^{++} concentrations greatly enhanced activity at higher (10^{-5} to 10^{-4} M) DCP levels. These results suggest that discrepancies in the literature concerning inhibition or enhancement of IAA destruction by Mn^{++} may be ascribed to the use of breis or enzyme preparations containing different levels of endogenous phenolic cofactor.

References

1. Galston, A. W. & Baker, R. S.: Studies on the physiology of light action III Light activation of a flavoprotein enzyme by reversal of a naturally occurring inhibition. — Amer. J. Bot. 38: 190. 1951.
2. — Bonner, J. & Baker, R. S.: Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. — Arch. Biochem. and Biophys. 42: 456. 1953.
3. — & Dalberg, L. Y.: The adaptive formation and physiological significance of indoleacetic acid oxidase. — Amer J. Bot. 41: 373. 1954.
4. Goldacre, P. L., Galston, A. W. & Weintraub, R. L.: The effect of substituted phenols on the activity of the indoleacetic acid oxidase of peas. — Arch. Biochem. and Biophys. 43: 358. 1953.
5. Gortner, W. A. & Kent, M.: Indoleacetic acid oxidase and an inhibitor in pineapple tissue. — J. Biol. Chem. 204: 593. 1953.
6. Kenten, R. H. & Mann, P. J. G.: The oxidation of certain dicarboxylic acids by peroxidase systems in presence of manganese. Biochem. J. 53: 498. 1953.
7. — The oxidation of indolyl-3-acetic acid by waxpod bean rootsap and peroxidase system. — Biochem. J. 59: 110. 1955.
8. — The oxidation of β-(3-indolyl)propionic acid and γ-(3-indolyl)-n-butyric acid by peroxidase and Mn^{2+}. — Biochem. J. 61: 353. 1955.

9. Lockhart, J. A.: The role of 2,4-dichlorophenol in the destruction of indoleacetic acid by peroxidase. — Plant Physiol. 30: 86. 1955.
10. Platt, R. S.: The inactivation of auxin in normal and tumorous tissues. — Ann. Biol. 30: 89. 1954.
11. Siegel, S. M. & Galston, A. W.: Peroxide genesis in plant tissues and its relation to indoleacetic acid destruction. — Arch. Biochem. and Biophys. 54: 102. 1955.
12. Tang, Y. W. & Bonner, J.: The enzymatic inactivation of indoleacetic acid I. Some characteristics of the enzyme contained in pea seedlings. — Arch. Biochem. and Biophys. 13: 11. 1947.
13. Wagenknecht, A. C. & Burris, R. H.: Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. — Arch. Biochem. and Biophys. 25: 30. 1950.
14. Walton, J. H. & Jones, D. O.: The catalytic decomposition of hydrogen peroxide in certain non-aqueous solutions. — J. Amer. Chem. Soc. 38: 1956. 1916.

The Influence of Calcium on Root Mitochondria

By

Cirl Florell

Botanical Laboratory, Lund
(Received February 16, 1956)

Introduction

The effect of calcium on the consumption of nitrate by wheat plants has been studied by Burström (2). He found that calcium increases the uptake of nitrate and could also show that it was primarily an increase in the absorption and not in the assimilation of nitrate. Nitrate is taken up in the same manner as other anions; according to the theory of Lundegårdh by means of the cytochrome-cytochrome oxidase system (Lundegårdh, 7). Stafford (13) and Millerd (8) have shown that cytochrome oxidase occurs in intracellular particles, presumably the mitochondria, and these results have been confirmed by other workers (cf. Davies, 4, Laties, 6). Results of Robertson *et al.* (12) confirm that the ion absorption mechanism is located to the mitochondria. Thus, if calcium can influence the formation of the mitochondria, this could explain the effect on the uptake of nitrate. The present study is an attempt to ascertain if calcium has any influence on the formation and activity of the mitochondria.

Methods

The test material was young plants of Weibull's Eroica wheat. The seeds were germinated for 36 hours on moist filter paper at $22°$ C and then transferred to 1 litre beakers containing 500 ml of the nutrient solution. The plants were grown under artificial illumination at $22°$ C in well aerated, stationary solutions for 4 days. The nutrient solution, changed after 2 days, had the following composition: KNO_3

1/200, KH_2PO_4 1/200, Na_2HPO_4 1/200, $MgSO_4$ 1/500, Fe-citrate 1/50000, $MnSO_4$ 1/100000, and traces of B, Zn, and Mo. All concentrations are expressed in mole per litre. Calcium was given as $CaCl_2$ at two levels, 10^{-4} M and 0, respectively. The pH was 6.9 in all series. Every series contained 200 plants per concentration of calcium.

Dry weights were determined after drying in vacuo at 50° C. *Nitrogen content* was determined by the Kjeldahl method in micro.

Fractioning procedure: 8—10 g fresh weight of the roots were homogenized in a Waring blendor at $+2$—$+4°$ C. Three fractions of the homogenate were prepared by a modification of the method of Stafford (13). The homogenate was centrifuged at $500\times$ g for ten minutes at $+4°$ C, without previous dilution. The precipitate, fraction a, contains the cell-wall material and the nuclei. The supernatant, containing the mitochondrial fraction, the cell-sap, and the soluble plasm, was made up to a volume of 50 ml. Part of this solution was used for respiration experiments, using the method of Warburg, and the rest was centrifuged for one hour at $18000 \times$ g. The precipitate yielded fraction b, which contained the mitochondria, while the supernatant, fraction c, was a clear solution of the cell-sap and the soluble plasm.

It was necessary to work quickly and to keep the temperature as low as possible, $+2$—$+4°$ C, or else the differences in the respiratory activity would be very difficult to measure owing to the inactivation of the enzymes concerned. All manipulations from harvesting the material up to the high-speed centrifuging had to be carried out within one hour to give good results.

Results of fractioning

All experiments were carried out at two levels of calcium, 10^{-4} and 0 moles per litre, respectively. When the experiments were stopped, the roots in the calcium-free solutions were healthy and only showed slight signs of calcium deficiency, such as impaired growth of root hairs and a faint red-brown colouring of the roots.

The distribution of dry matter and nitrogen of the two concentrations is shown in Table 1. The first two columns give the distribution of dry weight between the three fractions a, b, and c. Fraction a, consisting of cell-wall material chiefly, shows an increase by about 30 per cent with calcium. This increase must not necessarily be related to any effect of calcium on the mitochondria but may illustrate the effect that calcium exerts on the cell-elongation; according to Burström's hypothesis an influence on the growth by cell-wall formation (Burström, 1). Fraction b, composed of the mitochondria, is more than 50 per cent larger in the calcium series. The amount of fraction c is the same in the two series, that is, there is the same content of plasm per plant, and thus the increase of the mitochondria is entirely due to an effect of calcium.

The nitrogen content of fraction a is, of course, rather low and does not

Table 1. *Distribution of dry matter and nitrogen between the fractions.* Protein content calculated from Kjeldahl-N values multiplied by the factor 6.25.

Series number	Frac-tion	Dry weight of fractions per 200 plants		Ratio: + Ca/— Ca	N mmol per g dry weight of fractions		Protein of fraction *b*		
		— Ca	+ Ca		— Ca	+ Ca	% of dry weight		Ratio: + Ca/— Ca
							— Ca	+ Ca	
2	*a*	0.457	0.600	1.31	0.44	0.46			
	b	0.034	0.052	1.53	3.95	4.45	34.5	39.0	1.13
	c	0.134	0.073	0.55	0.67	0.70			
3	*a*	0.368	0.594	1.61	0.23	0.21			
	b	0.029	0.048	1.65	3.82	4.80	33.5	42.5	1.27
	c	0.324	0.250	0.77	1.25	1.37			
4	*a*	0.513	0.707	1.38	0.47	0.53			
	b	0.041	0.067	1.63	4.38	5.29	38.5	46.0	1.20
	c	0.206	0.236	1.14	1.07	1.27			
5	*a*	0.435	0.500	1.15	0.13	0.24			
	b	0.028	0.037	1.32	3.89	5.50	34.0	48.0	1.41
	c	0.272	0.312	1.14	0.63	0.66			
6	*a*	0.405	0.445	1.10	0.15	0.28			
	b	0.046	0.064	1.39	4.25	5.46	37.0	47.5	1.28
	c	0.259	0.286	1.10	0.70	0.71			
7	*a*	0.398	0.485	1.22					
	b	0.052	0.073	1.40	—	—	—	—	—
	c	0.235	0.212	0.90					
9	*a*	0.340	0.547	1.60	0.70	0.84			
	b	0.023	0.037	1.60	4.30	5.60	37.5	49.0	1.31
	c	0.267	0.220	0.82	1.03	1.12			
12	*a*	0.294	0.380	1.29	0.62	0.69			
	b	0.021	0.034	1.61	3.50	4.50	31.0	39.5	1.27
	c	0.206	0.210	1.02	0.88	0.93			
13	*a*	0.308	0.315	1.02	0.77	0.82			
	b	0.028	0.042	1.50	3.66	4.85	32.0	42.5	1.33
	c	0.224	0.273	1.22	0.96	1.10			
15	*a*	0.488	0.612	1.25	0.71	0.77			
	b	0.030	0.052	1.73	3.29	4.61	29.0	40.0	1.38
	c	0.470	0.535	1.14	1.09	1.16			
Mean	*a*	0.400	0.518	1.29 ± 0.06	0.47	0.54			
	b	0.033	0.051	1.54 ± 0.04	3.90	5.00	34.0 ± 1.05	43.5 ± 1.34	1.29 ± 0.03
	c	0.260	0.261	1.00 ± 0.07	0.92	1.00			

change very much with the calcium concentration. Fraction *c* is richer in nitrogen than *a* but also here there is no marked difference between the two series. The mitochondrial fraction, *b*, on the other hand, has a high content of nitrogen and the addition of calcium results in an increase by almost

30 per cent. If one calculates the percentage protein of the dry weight of fraction *b*, shown in the last two columns of the table, it is seen that 43.5 per cent, in the calcium series, and 34.0 per cent, in the calcium-free series, of the dry weight is made up of protein. Stafford (13) found that 30 to 40 per cent of the dry weight was protein in the particulate fraction, which most resembles fraction *b* in the present investigation.

Respiration experiments

The oxygen uptake was determined at 25° C in a Warburg apparatus according to the description of Umbreit *et al.* (15). 1.5 ml of the diluted solution containing fractions *b* and *c* was used in each Warburg flask. It was impossible to separate the two fractions for these tests, because the temperature was so high during the high-speed centrifuging that the enzyme systems were damaged, and thus, no reliable results could be obtained. The results given in Table 2 are based on two readings in all experiments. The oxygen uptake was linear over 120 minutes, readings being made every 15 minutes. The results are calculated per mg N of fraction *b*, containing the mitochondria. which, as Millerd and Bonner (10), and Millerd (9) have proved, are the site of many enzyme systems concerned with respiration. The results show that there is no significant difference in respiratory activity computed in this way between the two series. The respiration pertaining to the mitochondria calculated per plant is higher, of course, for the calcium series. This is evident from the results in Table 1; not only is the amount of mitochondria higher, but also the nitrogen content of these particles is increased with calcium, and the total increase in respiration is about 100 per cent.

Dry weight, fresh weight, nitrogen content, and root length data are sum-

Table 2. *The respiratory activity of wheat root homogenates.* The oxygen uptake is expressed as μl O_2 per hour per mg N of fraction *b*. 1.5 ml of fraction *b+c* were used in all experiments as enzyme solution. To this solution different substrates were added as shown in the last column. 0.3 ml 15 % KOH in the centerwell. The bath temperature was 25° C. Total flask volume 2.3 ml.

Series number	Endogenous		Glucose added 0.05 *M*		Ratio: +Ca/— Ca		Respiring system	pH
	— Ca	+ Ca	— Ca	+ Ca	Endog.	With gluc.		
6	430	480	670	620	1.11	0.93	Phosphate buffer	7.2
9	425	480	565	620	1.13	1.09	0.005 *M* glucose-6-phosphate	6.8
12	360	395	500	510	1.09	1.02	0.005 *M* fructose-6-phosphate	6.8
13	306	312	430	445	1.02	1.03	»	6.8
Mean	380	416	541	549	1.09	1.02	—	—

Table 3. *Summary of dry weight of the fractions, total dry weight, fresh weight, root length, nitrogen content, and the corresponding ratios.* Average of 8 to 10 values.

Dry weight of fractions per 200 plants g.		Total dry weight per 200 plants g.		Fresh weight per 200 plants g.		N mmol per g dry weight of fractions		Root length cm.		Ratio: +Ca/—Ca				
										Dry weight of fractions	N content of fraction b	Total dry weight	Fresh weight	Root length
—Ca	+Ca	—Ca	+Ca	—Ca	+Ca	—Ca	+Ca	—Ca	+Ca					
a 0.400	0.518					0.47	0.54			1.29				
b 0.033	0.051	0.692	0.830	12.17	15.32	3.90	5.00	8.9	11.7	1.54	1.29	1.20	1.26	1.32
c 0.260	0.261					0.92	1.00			1.00				

marized in Table 3. From this it can be seen that the calcium series have a higher water content, which is probably due to the effect calcium exerts on cell elongation; the amount of meristematic tissue is relatively smaller for roots with calcium. As already has been mentioned the increase in dry matter of fraction *a* can also be attributed to this effect of calcium; Table 3 shows that the increase of fraction *a* and of root length are about the same for the calcium series.

Discussion

The results presented above show that the amount of mitochondria is pronouncedly increased by calcium. As many of the effects that calcium exerts, such as nitrate uptake, cell elongation, and formation of mitochondria, ought to be energy requiring processes, one explanation could be that calcium in some way directly affects respiration and metabolism in general. However, the results in Table 2 show that the respiratory activity *per se* is not affected by this ion.

Dianzani (5) has shown that isolated mitochondria are very susceptible to changes in the osmotic environment. Their size and shape change with changes in the osmotic properties of the homogenizing medium. The respiratory activity is also affected. He suggests that the mitochondria in the intact cell may also be sensitive to osmotic changes in the cytoplasm and that the respiratory processes could be regulated in this way. Tedeschi and Harris (14) have also investigated the osmotic behaviour of mitochondria and their results show that the mitochondria possess a semipermeable membrane similar to the tonoplast. In Butler's opinion (3) the mitochondria and the tonoplast have similar functions. The vacuole and the mitochondrion are both able to perform osmotic work and in mature root cells the cytochrome oxidase may be located to the tonoplast. Robertson *et al.* (12) have studied the accumulation of salts in the mitochondria. They found that the mito-

chondria can accumulate anions against a concentration gradient; the accumulatory mechanism was presumably coupled to electron carriers of respiration.

We know that calcium regulates the permeability of the plasm membranes and for above-mentioned reasons it may have the same effect on the membrane of the mitochondrion. It is suggested as an explanation that calcium favours the formation of the mitochondria by means of its general influence on the organization of the cytoplasm. Such a direct effect of calcium on the formation of the mitochondria obviously could explain other effects of calcium, such as the increase in respiration per plant found in the author's experiments and the increase in nitrate absorption found by Burström (2). The increase in nitrate uptake shown in his material could entirely be explained as an effect of the higher amount of mitochondria with reference to Lundegårdh's (7) theory of anion absorption and a location of the active mechanism to the mitochondria according to Robertson *et al.* (12). However, this means that calcium, at least within certain ranges of concentration, would enhance the absorption of ions in general. Indications in this direction are found in the literature (cf. Overstreet *et al.*, 11, Viets, 16), but whether these results can be directly compared with ours, needs to be further elucidated. The effect of calcium on cell elongation, however, cannot be explained by an increase in the amount of mitochondria.

Summary

The influence of calcium on the mitochondria of wheat roots has been studied. An external calcium concentration of 10^{-4} mole per litre increased the amount of mitochondria by 54 per cent and their protein content by 29 per cent as compared with controls. The respiratory activity per mg protein of the mitochondria was not affected by calcium. The amount of other cytoplasmic materials was not affected either.

It is suggested that calcium favours the formation of mitochondria by means of its general influence on the organization of the cytoplasm.

The connexion between the mitochondria and the uptake of anions has been discussed with regard to the results obtained.

References

1. Burström, H.: Studies on growth and metabolism of roots. VIII. Calcium as a growth factor. — Physiol. Plant. 5: 391. 1952.

2. — Studies on growth and metabolism of roots. X. Investigations of the calcium effect. — Physiol. Plant. 7: 332. 1954.

3. Butler, G. W.: The connexion between respiration and salt accumulation. I. Preliminary note. — Physiol. Plant. 6: 662. 1953.

4. Davies, D. D.: The Krebs cycle enzyme system of pea seedlings. — J. Exp. Bot. 4: 173. 1953.

5. Dianzani, M. U.: On the osmotic behaviour of mitochondria. — Biochim. Biophys. Acta 11: 353. 1953.

6. Laties, G. G.: The dual role of adenylate in mitochondrial oxidations of a higher plant. — Physiol. Plant. 6: 199. 1953.

7. Lundegårdh, H.: Investigations as to the absorption and accumulation of inorganic ions. — Ann. Agr. Coll. Sweden 8: 234. 1940.

8. Millerd, A.: Succinoxidase of potato tuber. — Proc. Linn. Soc. N.S.W. 76: 123. 1951.

9. — Respiratory oxidation of pyruvate by plant mitochondria. — Arch. Bioch. and Biophys. 42: 149. 1953.

10. — & Bonner, J.: Oxidative phosphorylation by plant mitochondria. — Arch. Bioch. and Biophys. 42: 135. 1953.

11. Overstreet, R., Jacobson, L. & Handley, R.: The effect of calcium on the absorption of potassium by barley roots. — Plant Physiol. 27: 583. 1952.

12. Robertson, R. N., Wilkins, M. J., Hope, A. B. & Nesztel, L.: Studies in the metabolism of plant cells. X. Respiratory activity and ionic relations of plant mitochondria. — Aust. J. Biol. Sci. 8: 164. 1955.

13. Stafford, H. A.: Intracellular localization of enzymes in pea seedlings. — Physiol. Plant. 4: 696. 1951.

14. Tedeschi, H. & Harris, D.: The osmotic behaviour and permeability to non-electrolytes of mitochondria. — Arch. Bioch. and Biophys. 58: 52. 1955.

15. Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: Manometric techniques and tissue metabolism. — Burgess Publ. Co., Minneapolis. 1949.

16. Viets, F. G., Jr.: Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. — Plant Physiol. 19: 466. 1944.

Gallic Acid Isolated from Water Extracts of Litter from *Acer platanoides*

By

GERD BENDZ

Chemical Institute, University of Uppsala, Sweden
(Received February 17, 1956)

It has been reported (Melin and Wikén 1946) that cold water extracts of dried and ground autumn leaves of *Acer platanoides* L. show antibacterial activity when tested against *Staphylococcus aureus*. The present communication describes the investigation of litter extracts of *Acer platanoides,* the isolation of the antibacterial substance and its identification as gallic acid.

There was a great difference in the activity of the water extract prepared by the above authors and those obtained in this investigation. Melin and Wikén report inhibition zones of 21—23 mm, whereas the present author did only obtain zones of 10—12 mm. Experiments were consequently initiated to find out the reason for this discrepancy. As it could be possible, that the higher activity depended on a different concentration of some antibiotic agent other than gallic acid, a careful investigation of the litter extracts was carried out. However, it was proved, that the activity was due only to gallic acid or secondary products derived from it. Other possible explanations of the difference in activity could be, that a hypothetic inhibitor is present only in the litter of certain years or special localities. or that it does only appear during a very short time in the autumn. The investigation was extended along these lines. Litter were collected over a period of several years in different parts of Uppsala as well as from certain places about 10 kilometers outside Uppsala, cold water extracts thereof were prepared and tested. However, none of them had an inhibition zone greater than 12 mm. The same negative result was given by the investigation of litter collected at intervals of a few days in the autumn.

Table 1. *Effect of pH on the activity of gallic acid.*

pH	Inhibition zones in mm	
	Gallic acid	Standard solution
2.92	9.6	25.4
3.99	8.5	24.4
5.01	0	24.0
6.20	15.6	24.7
7.00	19.7	24.4

The assay values are average of 6 parallels.

During the investigation, it was discovered that the activity of gallic acid increased considerably when its water solution was neutralised with sodium hydroxide. An irreversible change occurs and the colourless aqueous solution turns deep green. The change in the activity with pH is shown in table 1. One per cent solution of gallic acid was used, and, as a standard, a penicillin solution containing 0.5 I.U. per ml.

It has long been known, that when gallic acid is treated with alkali in the presence of air, it is oxidised (cf. Schewket 1913). If the treatment is performed under special conditions, galloflavin $C_{12}H_6O_8$ is formed (Bohn and Graebe 1887, Haworth and McLachlan 1952). The structure of this compound has not yet been fully elucidated, but it is also sensitive to oxidation in alkaline media. These are the only information the author has been able to find concerning the oxidation products formed by gallic acid when treated with alkali, and the matter was not further investigated.

From table 1 it is evident, that the activity of gallic acid is rather low but that it is increased when gallic acid is mixed with some of its oxidation products. This might possibly be due to an intermediate substance, perhaps galloflavin, as it has also been found that there are some differences in activity, if aqueous solutions of gallic acid are neutralised to pH 7, or if gallic acid is first dissolved in sodium hydroxide and then neutralised to pH 7; the first mentioned solutions being always more active.

A quite satisfactory explanation of the observed difference in activity of the litter extracts has not been found; however, it might be possible that the higher activity is due to a pH effect. The extracts prepared by the author had a pH of about 4.0—4.5, at which pH, according to table 1, the activity of gallic acid is close to its minimum value. The increase of activity reported by Melin and Wikén to occur when extracts were autoclaved, may perhaps be due to the formation of more oxidation products. Other chemical changes can also influence the activity, as gallic acid is destroyed when autoclaved or boiled in aqueous solutions, pyrogallol being formed (Widmer 1929).

Gallic acid commonly occurs in higher plants, usually in form of tannin glycosides, and as such it has been isolated from other species of Acer, viz. *Acer aizuenes* (Tsujimoto 1951) and *Acer ginnala* (Perkin and Uyeda 1922). As in the case of most phenol compounds, the antibacterial activity of gallic acid has long been known. In recent years ethyl gallate, isolated from the leaves of *Haematoxylon campechianum* (Little *et al.* 1953), has been found to be active against *Mycobacterium tuberculosis*. Also in this case, oxidation products seem to play an important role, for ethyl gallate has optimal activity at pH 6.4.

Experimental

The leaves were collected from the ground in the autumn when their colour had turned yellow-brown; they were air-dried, ground and thoroughly mixed with distilled water in the proportion 1 : 5. The mixture was placed in a refrigerator for about 20 hours and was then filtered. The filtrate which contains about 2 per cent of solid material and had a pH of about 4.0—4.5 was mixed with 2 per cent by weight of Norite, which adsorbs all the active material, and left over night. The next day it was centrifuged, and the Norite was washed with a small quantity of water. The active material was eluted from the Norite with 80 per cent aqueous acetone, and from the eluate the acetone and some of the water were evaporated. The resulting solution was acidified with hydrochloric acid, saturated with sodium chloride and shaken with ethyl acetate which removed the active material. By concentrating the ethyl acetate to a small volume and adding petroleum ether, the active material was precipitated as a yellow powder. This was further purified by dissolving in ethyl acetate, treating with Norite and reprecipitating with petroleum ether. The precipitate was recrystallised with hot water and separated as fine colourless needles which, when heated in a capillary tube, decomposed without melting at about 220°. The elementary analysis gave C 49.74 and H 3.67 per cent and the electrometric titration an equivalent weight of 170.9. Gallic acid, whose formula $(C_7H_6O_5)$ requires C 49.42 and H 3.56, has an equivalent weight of 170.1. The ethyl ester of the isolated acid was also prepared; it melted at 154° and when mixed with authentic ethyl gallate, there was no depression in the melting point.

As there was a slight possibility, that some other antibacterial substance could be present in the dried leaves, aqueous litter extract as well as alcohol and acetone extracts thereof were thoroughly investigated by means of chromatographic adsorption, distillation with steam, extractions with different organic solvents and other techniques of separation. It was, however, found, that all active solutions contained gallic acid, and that all activity could be traced back to that acid.

In order to find out whether the litter extract could possibly have optimal activity only during a very short time in the autumn, leaves were collected at intervals of a few days over a period of three weeks partly from the ground and partly from a selected tree. Some of the leaves were air-dried, ground and prepared as above, others were, without drying, treated with distilled water in the proportion 1 : 5 in a turmix desintegrator; the mixture was then filtered and tested. The results are presented in table 2.

Table 2. *Inhibition zones in mm. on different days.*

Material		Date October					
		1	4	8	10	15	24
leaves from the ground	I	+[1]	+	9.2	9.3	9.7	7.3
	II	+	+	+	+	10.5	
leaves from the tree	I	+	7.7	+	8	10	
	II	+	+	+	+	7.7	

I = air-dried leaves.

II = leaves treated in the desintegrator.

[1] inhibition only within the test cylinder (diameter 7 mm).

Summary

Gallic acid has been isolated from litter extracts of *Acer platanoides*. It has low activity when tested against *Staphylococcus aureus*, but the activity is increased when gallic acid is neutralised with alkali. The optimal activity is found at pH 7 in aqueous solutions and may depend on the presence of some oxidation products formed when gallic acid is treated with alkali.

References

Bohn, R. & Graebe, C.: Über Galloflavin. — Ber. deutsch. Chem. Ges. 20: 2327. 1887.

Haworth, R. D. & McLachlan, J. M.: Galloflavin. — J. Chem. Soc. p. 1583. 1952.

Little, J. E., Foote, M. W., Rogers, W. I. & Johnstone, D. B.: Ethyl gallate, a Mycobacteria-specific antibiotic isolated from Haematoxylon campechianum. — Antibiotics & Chemotherapy 3: 183, 203. 1953.

Melin, E. & Wikén, T.: Antibacterial Substances in Water Extracts of Pure Forest Litter. — Nature 158: 200. 1946.

Perkin, A. G. & Uyeda, Y.: Occurrence of a crystalline tannin in the leaves of the Acer ginnala. — J. Chem. Soc. 121: 66. 1922.

Schewket, O.: Über die Oxydation von Gallussäure and Gallusgerbsäure (Tannin) an der Luft in Gegenwart von Alkalien und über eine Farbenreaktion von Blei. — Biochem. Z. 54: 277. 1913.

Tsujimoto, M.: Chemical components of the leaves of Acer aizuense. — Chem. Abstr. 47: 5498. 1953.

Widmer, O.: Über Bildung und Zerfall der Gallussäure, Pyrogallolkarbonsäure, Phloroglucinkarbonsäure und ihrer Kaliumsalze. — Z. physik. Chem. A 140: 161. 1929.

Phosphorylation Accompanying Succinate Oxidation by Mitochondria from Cauliflower Buds and Mung Bean Seedlings

By

G. Fritz and Aubrey W. Naylor

Department of Botany and Plant Pathology, The Pennsylvania State University
and Department of Botany, Duke University
(Received February 18, 1956)

Since the first demonstration (Millerd et al., 1951; Maruo and Matsuhashi, 1952) of the ability of mitochondria from higher plants to catalyze the incorporation of orthophosphate into adenosine triphosphate (ATP) during the oxidation of acids of the citric acid cycle, evidence has accumulated in support of the original observation (e.g., Conn and Young, 1955; Laties, 1953). However, factual information pertaining to possible differences in properties of mitochondria from various plant tissues is meager and details concerning the sequence of events involved in oxidative phosporylation by plant mitochondria are lacking.

During the course of an investigation of succinate oxidation by particulate enzymes from plant tissue, certain differences were found in the phosphorylating properties of enzyme suspensions prepared from cauliflower buds and mung bean seedlings and these are recorded here. The extent of fixation of inorganic phosphate in ATP was measured by glucose-6-phosphate (G-6-P) production induced by the addition of glucose, magnesium and hexokinase to the reaction mixture, and G-6-P was measured spectrophotometrically by the triphosphopyridine nucleotide glucose-6-phosphate dehydrogenase system.

Materials and Methods

Preparation of enzyme suspension. The topmost 2 to 3 mm. of flower buds of cauliflower inflorescences (*Brassica oleracea*) and entire 1-day-old seedlings of ger-

minating mung bean (*Phaseolus aureus*) were used as enzyme sources. Cauliflower heads were obtained from local markets. Mung bean seeds (Reuter Seed Co., New Orleans, Louisiana) were soaked in water for 6 hours and then planted in moist vermiculite (heat-expanded mica); germination in vermiculite proceeded at 21 to 23° C for 20 hours and primary roots of seedlings averaged 3 to 6 mm. in length at harvest. All plant material was washed in running tap water, dried with paper toweling, and stored at 0 to 4° C for about 3 hours.

Particulate enzyme suspensions (herein called mitochondria) were prepared by grinding 15 grams of plant tissue with 35 ml. of ice-cold solution consisting of 0.5 M sucrose and 0.1 M KH_2PO_4 at pH 7.3 in a previously-chilled mortar and pestle; a small quantity of cold reagent-grade white sand was sprinkled on the tissue to facilitate grinding. All solutions and containers used in the preparation of mitochondria were ice-cold and all manipulations, including centrifugation, were conducted at 0 to 4° C.

The slurry obtained by grinding was strained through one layer of cheesecloth and cleared of debris by centrifuging for 10 minutes at 500×gravity. Following decantation, the liquid was centrifuged for 30 minutes at 20,000×gravity in an International PR-2 refrigerated centrifuge. The resulting supernatant was discarded and the sediment was suspended — with the use of a power-driven plastic pestle — in 10 ml. of grinding solution. After a second 30-minute centrifugation at 20,000× gravity, the supernatant was discarded and the mitochondrial particles were suspended in 2 to 4 ml. of a solution consisting of 0.4 M sucrose, 0.02 M KH_2PO_4, 0.02 M glucose and 0.005 M $MgCl_2$ at pH 7.2. After an aliquot was taken for subsequent nitrogen determination (Thompson and Morrison, 1950), commercial hexokinase (5 mg. per ml. of mitochondrial suspension) was added and the mixture homogenized in a test tube with a snug-fitting power-driven plastic pestle. The mitochondrial suspension was used for experimentation without delay.

Anaerobic experiments. Succinate oxidation was carried out anaerobically in Thunberg tubes at room temperature (21 to 27° C.) and ferricyanide was used as the final electron acceptor. The main arm of the Thunberg tube contained the mitochondrial suspension, cytochrome c, ferricyanide and citraconic acid and was placed in an ice-water bath. After evacuation for 20 minutes, accompanied by vigorous shaking and tapping to remove air bubbles, the tubes were removed from the bath and succinate, adenosine diphosphate and muscle adenosine-5-phosphoric acid, contained in the side-arm, were tipped in to start the reaction; the total volume of the reaction mixture was 2.5 ml. Because ferricyanide interfered in the subsequent enzymatic determination of G-6-P, the reaction was allowed to continue until the yellowish color of ferricyanide disappeared and was replaced by the pinkish hue of reduced cytochrome c; this required about 2 hours for mung bean and about 3 hours for cauliflower. After incubation for the required time, the tubes were placed in boiling water for several minutes. (Upon opening, the sudden entrance of air was distinctly audible.) About 1/4 to 1/2 ml. water was added to replace that lost by evaporation; following centrifugation, the cleared solution was analyzed for G-6-P.

Aerobic experiments. Measurement of oxygen consumption was made in standard Warburg respirometers at 25° C; the gas phase was air and the center-well contained KOH-saturated paper. After a 10-minute equilibration period, succinate was tipped in from the side-arm of Warburg flasks; the total volume of the reaction mixture was 2.5 ml. Oxygen absorption was recorded at 5-minute intervals for 30 minutes.

The reaction was stopped by high-speed centrifugation; oxygen absorption during the time interval between opening the manometer flask and centrifugation was estimated by extrapolation. In certain aerobic experiments not involving measurement of oxygen consumption, reagents and mitochondria were mixed directly in plastic centrifuge tubes, and the reaction was terminated either by high-speed centrifugation or by addition of an equal volume of 2 N HCl followed by neutralization with 2 N NaOH. Trichloracetic acid, often used to stop enzymatic reactions, was avoided because it had an inhibitory effect on G-6-P dehydrogenase.

Analysis for G-6-P. Glucose-6-phosphate was determined spectrophotometrically with triphosphopyridine nucleotide (TPN) and G-6-P dehydrogenase, according to the techniques developed by Kornberg (1950) and Slater (1953 a, 1953 b). The enzymatic production of reduced-TPN was followed at 340 mμ with a Beckman Model DU spectrophotometer equipped with a photomultiplier. The micromoles of reduced-TPN produced in the cuvette equalled the micromoles of G-6-P in the sample and was obtained by multiplying the change in optical density by the factor 0.483, derived from the Lambert-Beer law and the extinction coefficient of reduced-TPN (Horecker and Kornberg, 1948).

Sources of cofactors and enzymes; abbreviations. Special chemicals were obtained from commercial sources as follows: triphosphopyridine nucleotide (TPN), glucose-6-phosphate dehydrogenase, adenosine diphosphate (ADP), muscle adenosine-5-phosphoric acid (AMP-5) and cytochrome c from Sigma Chemical Co.; glucose-6-phosphate (G-6-P) and adenosine triphosphate (ATP) from Schwarz Laboratories; hexokinase from Mann Research Laboratories; liver coenzyme concentrate and bovine plasma albumin (Armour and Co.).

Results

Stability of G-6-P. Since G-6-P was the end-product of the phosphorylation reaction, it was necessary to establish its degree of stability in the reaction mixture. When known amounts of spectrophotometrically-analyzed G-6-P were incubated at room temperature in the presence of mitochondria and with or without accessory substances such as ADP, AMP-5, cytochrome c, sucrose, glucose, citraconate, magnesium, phosphate and hexokinase, the recovery of G-6-P varied from 97 to 102 percent; incubation time was 4 hours with cauliflower mitochondria and 2 hours with mung bean mitochondria. From these results, it could be concluded that G-6-P was completely stable in the reaction medium.

Adenylate kinase. Evidence for the existence of adenylate kinase (myokinase) in cauliflower mitochondria was obtained during the course of testing the methods. (In a recent preliminary report, Mazelis and Stumpf (1955) indicated that this enzyme may be detected in mitochondria from other plant sources.) Adenylate kinase catalyzes the reaction (a) $2\,\text{ADP} \rightleftarrows \text{ATP} + \text{AMP}$. When 2 to 3 micromoles of spectrophotometrically-analyzed ATP were incubated for one hour at pH 7.2 with cauliflower mitochondria

in the presence of 0.02 M KH_2PO_4, 0.005 M $MgCl_2$, 0.02 M glucose, 0.4 M sucrose and hexokinase (5 mg. per ml. mitochondrial suspension), the micromoles of G-6-P produced equalled twice the number of micromoles of ATP initially added, conforming to the net reaction (b) ATP+2 glucose → 2 G-6-P+ AMP. Reaction (b) is the resultant of the adenylate kinase reaction (a) and the hexokinase reaction (c) ATP+glucose → ADP+G-6-P, as follows:

$$2 \text{ ATP} + 2 \text{ glucose} \rightarrow 2 \text{ ADP} + 2 \text{ G-6-P}$$
$$\underline{2 \text{ ADP} \rightleftharpoons \text{ATP} + \text{AMP} }$$
$$\text{ATP} + 2 \text{ glucose} \rightarrow 2 \text{ G-6-P} + \text{AMP}$$
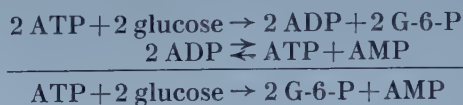
When ADP (the percent purity of which was assumed to be equal to that of ATP) was used instead of ATP, the micromoles of G-6-P produced were virtually equal to the number of micromoles of ADP initially added, in accordance with reaction (d) ADP+glucose → G-6-P+AMP, which is the resultant of equations (a) and (c). Cyanide (0.01 M) did not inhibit these reactions, indicating that oxidative phosphorylation due to the possible presence of residual substrates was not involved. Application of known inhibitors of adenylate kinase such as NaF and AMP (Slater, 1953 b) reduced the amount of G-6-P produced. These findings provided evidence that adenylate kinase was present and confirmed, with plant material, Slater's (1953 b) observations that ATP formed during oxidative phosphorylation in the presence of glucose, magnesium and hexokinase can be recovered quantitatively as G-6-P.

Adenylate kinase correction. In order to determine the extent of oxidative phosphorylation, G-6-P formed in the absence of added substrate by non-oxidative phosphorylation involving adenylate kinase and hexokinase must be subtracted from the G-6-P produced upon the addition of substrate. In all oxidative phosphorylation experiments reported here, this correction was determined in separate control experiments and applied (see Table 1). In the case of Thunberg experiments, the extent of the non-oxidative phosphorylation was determined under anaerobic conditions in the absence of ferricyanide. To reduce the "adenylate kinase correction", AMP was added in concentrations equal to that of ADP.

P/O ratios. The yield of oxidative phosphorylation (moles of inorganic orthophosphate esterified per atom of oxygen consumed) during the aerobic oxidation of succinate to fumarate has a probable value of 2 for particulate enzymes from animal tissues (Lehninger, 1955). In a recent preliminary report, Conn and Young (1955) obtained values approaching 2 for succinate oxidation by plant mitochondria from white lupine seedlings. Using the methods described above, P/O ratios approaching 2 were readily obtained when succinate was oxidized aerobically by mitochondria from mung bean seedlings

Table 1. *Phosphorylation associated with oxidation of succinate or ascorbate, with oxygen or ferricyanide as electron acceptors.* The reaction mixtures contained 1.21×10^{-5} M added cytochrome c, 0.001 M ADP, 0.001 M AMP-5, 0.4 M sucrose, 0.02 M KH_2PO_4, 0.02 M glucose, 0.005 M $MgCl_2$, 2.5 mg. hexokinase, 0.5 ml. mitochondrial suspension; in the aerobic experiments, 0.02 M succinate (together with 0.04 M citraconate) or 0.02 M ascorbate, were used; for the anaerobic experiments with ferricyanide, 0.01 M succinate, 0.02 M citraconate and 0.01 M $K_3Fe(CN)_6$ were used. Total volume, 2.5 ml.; pH 7.2. KOH-saturated paper was placed in the center-well in Warburg experiments. See text for details concerning measurement of G-6-P and "adenylate kinase correction" and duration and temperature of experiments. Note: phosphorylation values recorded here are the maximal values achieved.

Source of mitochondria	Substrate	Oxidant	N content per vessel (mg)	Total G-6-P produced (μ moles)	Adenylate kinase correction (μ moles)	ΔP $(=\Delta$G-6-P$)$ (μ moles)	ΔO (μ atoms)	$(Fe(CN)_6)^{-3}$ (μ moles)	P/O or $\dfrac{P}{2(Fe(CN)_6)^{-3}}$
Mung Bean	Succinate	Oxygen	2.1	12.35	1.82	10.53	5.58	—	1.89
Cauliflower	»	»	1.2	7.71	1.69	6.02	6.54	—	0.92
Mung Bean	»	Ferricyanide	2.2	3.58	1.21	2.37	—	25.00	0.19
Cauliflower	»	»	1.3	0.98	1.06	zero	—	25.00	zero
Mung Bean	Ascorbate	Oxygen	2.2	4.41	1.16	3.25	6.13	—	0.53
Cauliflower	»	»	0.6	1.90	1.32	0.58	4.82	—	0.12
Cauliflower (pretreated in distilled water)	»	»	0.6	2.73	0.56	2.17	7.02	—	0.31

(see Table 1). However, P/O ratios in similar experiments with cauliflower mitochondria were consistently below 1 (Table 1). Citraconic acid, a competitive inhibitor of fumarase (Jacobsohn, 1953) was added in concentrations equal to twice that of succinate in order to isolate the succinate-fumarate reaction. The ability of the enzyme suspensions to catalyze oxygen absorption upon the addition of fumarate was very small; when fumarate instead of succinate (at the same concentrations) was added to cauliflower or mung bean mitochondria, the oxygen uptake was about $^1/_{25}$th of that induced by succinate.

Mitochondria from both plant sources responded similarly to cyanide; experiments with cyanide were performed in Warburg flasks in which KOH-saturated paper was omitted from the center-well. Application of increasing concentrations of cyanide (10^{-5} M to 10^{-2} M) resulted in progressive decreases in oxygen absorption and in phosphorylative capacity and these decreases paralleled one another closely. At 0.01 M cyanide, the percentage decreases in oxygen uptake and in phosphorylation were over 90 percent.

It is possible that a natural inhibitor of phosphorylation during succinate oxidation may be present in cauliflower mitochondria. Thus, when a mito-

chondrial suspension from cauliflower was mixed with mung bean mito-
chondria, the P/O ratio for succinate oxidation was lower than that with
mung bean mitochondria alone.

Site of phosphorylation with respect to cytochrome c. In view of the dis-
crepancy between P/O ratios obtained with mitochondria from mung bean
seedlings and cauliflower, further experiments were performed to study the
site of phosphorylation with respect to cytochrome c. Lehninger (1955)
reviewed the evidence relating to the ability of animal mitochondria to
catalyze the incorporation of inorganic phosphate into ATP during the oxida-
tion of reduced cytochrome c by oxygen. Similar data were sought for cauli-
flower and mung bean mitochondria. Oxidation of cytochrome c by oxygen
in the presence of ascorbic acid, which reduces cytochrome c non-enzymati-
cally, was accompanied by G-6-P production in the presence of mitochondria
from both sources (Table 1). For cauliflower, it was found that virtually no
oxidative phosphorylation occurred in the absence of added cytochrome c.
Increased production of G-6-P was obtained by pretreatment of cauliflower
mitochondria in distilled water for 30 minutes (see Table 1). Pretreatment
consisted of washing the mitochondria in ice-cold distilled water during the
second high-speed centrifugation. Lehninger (1955) postulated that hypo-
tonic pretreatment of mitochondria leads to alterations in structure or per-
meability; presumably this treatment could make possible the entry of reduced
cytochrome c, formed externally by non-enzymatic reduction with ascorbate,
to the internal regions of the mitochondria where phosphorylation presum-
ably takes place.

When measuring P/O ratios with ascorbate and cytochrome c. oxygen
absorption was corrected by subtracting a boiled blank. Even so. the P/O
ratios were markedly below one (see Table 1). This may be explained, at
least in part, by assuming that oxidation of reduced cytochrome c by oxygen
may be catalyzed independently of phosphorylation. Thus, oxidation of
cytochrome c might be achieved by cytochrome oxidase present at the sur-
face of mitochondria and not linked to phosphorylating enzymes, or by
fragments of mitochondria which contain cytochrome oxidase but do not
have phosphorylating ability (Lehninger, 1955).

Phosphorylation accompanying succinate oxidation by cytochrome c (in
the absence of atmospheric oxygen) can be determined experimentally by
incubating mitochondria with ferricyanide, which oxidizes cytochrome c
non-enzymatically, i.e., acts as an electron acceptor. Using this technique,
mitochondria from animal tissues have been shown to induce phosphoryla-
tion in manometric experiments where a nitrogen-carbon dioxide mixture
was used as the gas phase (Copenhaver and Lardy, 1952; Cross et al., 1949).
Also, McEwen and Eiler (1950) obtained evidence for phosphorylation in

rat brain homogenates, under anaerobic conditions, with a platinum electrode as electron-acceptor and a ferro-ferricyanide couple as mediator. With mitochondria from mung bean seedlings, $P/2(Fe(CN_6))^{-3}$ ratios (estimated on the basis of complete reduction of ferricyanide) approaching 0.2 were readily obtained under anaerobic (Thunberg) conditions (see Table 1). In these experiments, it was necessary to incubate the reaction mixture for about two hours at room temperature, after evacuation and mixing, to bring about the complete disappearance of ferricyanide, which interfered in the subsequent analysis for G-6-P. The low value of the ratio may possibly be due to toxic effects exerted by ferricyanide on the mitochondrial enzymes, as suggested by Hunter (1951).

In contrast, despite numerous trials, no G-6-P production due to oxidative phosphorylation ever occurred under anaerobic conditions when mitochondria from cauliflower were used (Table 1) and this was true even though the catalytic reduction of ferricyanide took place. Complete disappearance of the yellowish color of ferricyanide and its replacement by the pinkish hue of reduced cytochrome c required about 3 hours incubation at room temperature. Among the changes in procedure tried without success were: a) additions to the reaction mixture such as boiled tomato leaf extract, liver coenzyme concentrate, bovine plasma albumin; b) changes in the preparative grinding and washing solutions such as addition of 0.01 M ethylenediaminetetraacetic acid or omission of phosphate or a third highspeed centrifugation of mitochondria in a solution of 0.5 M sucrose plus 0.001 M KCl; c) changes in the reaction solution such as use of 0.5 M sucrose instead of 0.4 M sucrose; d) pretreatment of mitochondria in distilled water; c) rapid homogenization of plant material in a high-speed blendor (Omni-Mix) instead of with mortar and pestle.

Stability of mitochondrial preparations. It was found that differences in enzymatic stability existed between mitochondrial preparations from mung bean seedlings and cauliflower. Incubation of mung bean mitochondria in a ice-water bath for one hour before addition to the reaction vessel affected neither the oxygen absorbing ability nor the phosphorylating capacity during aerobic oxidation of succinate. The measured P/O ratio was the same as that found for mitochondria used immediately after preparation. But when mitochondrial enzyme preparations from cauliflower were treated in a similar manner, the rate of oxygen absorption was decreased 3 percent and the production of G-6-P was 23 percent lower. Consequently the P/O ratio was decreased almost 20 percent. (After incubation for 2 hours in an ice-water bath, the phosphorylating capacity of cauliflower mitochondria during aerobic succinate oxidation was 50 percent of that of the enzyme suspension used immediately after preparation.) Decline in ability of cauliflower mito-

chondria to phosphorylate in the presence of oxygen and succinate after incubation at low temperature, combined with the use of the fumarase inhibitor, citraconate, in the present experiments, may account for the fact that the highest P/O ratios obtained here with cauliflower, nearly 1 (see Table 1), were lower than the highest (1.42) reported by Laties (1953). A further possible cause of difference in results may be traced to differences in methods of measuring phosphorylation; Laties used disappearance of inorganic phosphate as a measure of incorporation into ATP.

Discussion

Lewis and Slater (1954) studied the ability of particulate enzymes from insects (blowflies) to catalyze the incorporation of inorganic phosphate into ATP during the oxidation of α-ketoglutaric acid, and found that the P/O ratios obtained were considerably below those achieved under identical conditions with enzymes from rat heart. Also, the present paper reports that mitochondria prepared from two different plant species by identical extraction procedure exhibited marked differences in phosphorylating ability during succinate oxidation, when the extent of fixation of inorganic phosphate in ATP was measured by the same method.

Lehninger's (1955) discussion leads to the conclusion that succinate oxidation by animal mitochondria is accompanied by two phosphorylative acts, one occurring during the passage of electrons from succinate to cytochrome c and the other occurring during the passage of electrons from cytochrome c to molecular oxygen. The results presented here strongly support this point of view for mitochondria from mung bean seedlings. But, the findings that cauliflower mitochondria catalyzed phosphorylation during aerobic oxidation of cytochrome c in the presence of ascorbic acid and did not catalyze phosphorylation during the anaerobic oxidation of succinate, coupled with the finding that the maximum P/O ratio for aerobic succinate oxidation by these mitochondria was slightly less than 1; — these findings lead to the supposition that either the enzyme complex responsible for incorporation of inorganic phosphate into ATP during anaerobic oxidation of succinate by cytochrome c and ferricyanide is lacking in cauliflower or that these phosphorylating enzymes are characterized by a marked lability or sensitivity to damage upon extraction. (In this connection, also see Lewis and Slater, 1954.) The latter alternative may appear more credible in view of the results obtained with mung bean mitochondria, for which the maximal ratios achieved during anaerobic succinate oxidation were much lower (0.19) than those obtained during aerobic oxidation of cytochrome c in the presence

of ascorbic acid (0.53) (see Table 1). However, in assessing the significance of the negative results for the anaerobic cauliflower experiments, it must be borne in mind that more than one factor may be operative and it would be difficult to evaluate the relative effects of these factors; for example, it is possible that a high degree of susceptibility to toxicity by ferricyanide together with inherent lability of the enzymes may account for the absence of phosphorylation.

The available evidence does not provide a final answer to the question of whether failure to phosphorylate during the anaerobic enzymatic oxidation of succinate by ferricyanide is an inherent property of cauliflower mitochondria or is due to instability of the phosphorylating enzymes. Also, it cannot be concluded at present that the yield of phosphorylation in cauliflower mitochondria, *in vivo*, is lower than in mung bean seedlings.

Summary

A study was made of the ability of particulate enzyme preparations from cauliflower and from 1-day mung bean seedlings to phosphorylate ADP during the oxidation of succinate in the presence of the fumarase inhibitor, citraconate. P/O ratios approaching 2 were found for mung bean mitochondria, whereas the ratios obtained with cauliflower mitochondria were less than one. Oxidative phosphorylation was demonstrated by mitochondria of both species when ascorbate was used as substrate in the presence of added cytochrome c. In anaerobic experiments with succinate and added cytochrome c, in which ferricyanide was employed as final electron-acceptor, only the mitochondria from mung bean seedlings induced oxidative phosphorylation, although both sources of mitochondria catalyzed the disappearance of ferricyanide.

References

Conn, E. E. & Young, L. C. T.: Oxidative phosphorylation in lupine mitochondria. — — Federation Proc. 14: 195. 1955.
Copenhaver, Jr., J. H. & Lardy, H. A.: Oxidative phosphorylation: pathways and yield in mitochondrial preparations. — Jour. Biol. Chem. 195: 225. 1952.
Cross, R. J., Taggert, J. V., Covo, G. A. & Green, D. E.: Studies on the cyclophorase system. VI. The coupling of oxidation and phosphorylation. — Jour. Biol. Chem. 177: 655. 1949.

Horecker, B. L. & Kornberg, A.: The extinction coefficients of the reduced band of pyridine nucleotides. — Jour. Biol. Chem. 175: 385. 1948.

Hunter, Jr., F. E.: Oxidative phosphorylation during electron transport. — McElroy, W. D. & Glass, B.: Phosphorus Metabolism, Vol. 1, p. 297. The Johns Hopkins press. 1951.

Jacobsohn, K.: Sur l'inhibition compétitive de la fumarase. — Enzymologia 16: 113. 1953.

Kornberg, A.: Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. — Jour. Biol. Chem. 182: 779. 1950.

Laties, G. G.: The physical environment and oxidative and phosphorylative capacities of higher plant mitochondria. — Plant Physiol. 28: 557. 1953.

Lehninger, A. L.: Oxidative phosphorylation. — The Harvey Lectures, 1953—1954, p. 176. The Academic press. 1955.

Lewis, S. E. & Slater, E. C.: Oxidative phosphorylation in insect sarcosomes. — Biochem. Jour. 58: 207. 1954.

Maruo, B. & Matsuhashi, M.: Oxidative phosphorylation in higher plant homogenate. — Jour. Agri. Chem. Soc. (Japan) 26: 440. 1952.

Mazelis, M. & Stumpf, P. K.: Particulate adenylic kinase in higher plants. — Federation Proc. 14: 253. 1955.

McEwen, W. K. & Eiler, J. J.: Enzymatic anodic phosphorylation in rat brain homogenates. — Federation Proc. 9: 200. 1950.

Millerd, A., Bonner, J., Axelrod, B. & Bandurski, R. S.: Oxidative and phosphorylative activity of plant mitochondria. — Proc. Natl. Acad. Sci. (U.S.) 37: 855. 1951.

Slater, E. C.: Spectrophotometric determination of fructose-1,6-diphosphate, hexosemonophosphates, adenosinetriphosphate and adenosinediphosphate. — Biochem. Jour. 53: 157. 1953 a.

— A method for measuring the yield of oxidative phosphorylation. — Biochem. Jour. 53: 521. 1953 b.

Thompson, J. F. & Morrison, G. R.: Determination of organic nitrogen. — Anal. Chem. 23: 1153. 1951.

Influence of Time of Sowing on the Effect of 2,4-D on Growth and Maturity of Wheat

By

S. N. Bhardwaj and I. M. Rao

Department of Botany, Agra College, Agra, India
(Received February 25, 1956)

No other synthetic phytohormone has received in recent years as much attention as 2,4-Dichlorophenoxyacetic acid (2,4-D) chiefly due to its selective herbicidal nature. The literature relating to its selective toxicity is extensive, but little attention seems to have been paid to its stimulatory effect on growth and maturity of crop plants. In an earlier preliminary investigation, the authors (1955) observed that the synthetic hormones, NAA and 2,4-D, particularly the latter, were beneficial to growth and maturity of wheat, when the seeds were given a pre-sowing soaking treatment in 10 ppm. of the hormone solution for 24 hours; but the sowing was delayed till December, and thus, it was felt that the treatment-effect should be studied for the normal November-sowing too. The present paper deals with effect of presowing soaking of wheat seeds in 10 ppm. and 100 ppm. of 2,4-D on growth and maturity when the seeds were sown in the first week of November and of December.

Review of Literature

As already mentioned, studies on 2,4-D related chiefly to its selective herbicidal nature. The differential toxicity of the chemical to cereals and dicotyledonous weeds has been utilised to fix the optimum dose, by actual experimentation in each case, which will minimise the weed-growth with minimum injury to the crop plant. Quoting a single recent reference out of the extensive literature, Pande (1954) observed that wheat crop infested with weeds gave almost as much yield of grain, when sprayed with 3.25 lb. per acre of sodium dichlorophenoxyacetate, as when the

17

plots were hand-weeded; higher doses resulted in better eradication of weeds but gave lower yields, apparently due to the toxic effect on wheat plants; still lower doses were not tried; further, spraying at the tillering stage was more beneficial than at the boot or blossom-stage. The experiment, however, does not give an idea of the independent effect of 2,4-D on wheat plants, as, in studies on crop plants, 2,4-D is used mainly as a weedicide.

Regarding the effect of 2,4-D on germination and growth of plants, different methods have been adopted for the application of the substance such as spraying, addition to the soil and pre-sowing treatment of seeds. Mitchell and Marth (1945) studied the effect of spraying different concentrations of 2,4-D on the growth of grasses; they observed an initial depression in growth followed by a gradual recovery. Allard et al. (1946) added 2,4-D to the soil and observed inhibition of germination of wheat and oats. Sen and Moolani (1954) noted an acceleration in germination and seedling growth of rice when the seeds were treated with 0.1 to 10 ppm. of 2,4-D; higher concentrations depressed both. Hamner et al. (1946) gave presowing soaking treatment to seeds of various crop plants and weeds for four hours in 1, 10 and 100 parts per litre of 2,4-D solution; germination and seedling-top-growth were slightly inhibited by the highest concentration; the lower doses showed no effect. Stromme and Hamner (1948) noted increase in auxillary growth as well as in number of pods per plant, when bean plants were sprayed with 1 or 10 ppm. of sodium 2,4-dichlorophenoxyacetate.

In recent years, greater attention has been paid to the mode of action of 2,4-D on plant metabolism. It is sufficient to mention here that Rebstock et al. (1954), while studying the effect of 2,4-D on P-metabolism of Cranberry bean plants, noted a doubling of the nucleic-acid content of the stem as a result of the treatment; they attributed to it the unusual growth and development of the treated plants.

Persistence of 2,4-D in remote regions of the plant, long after the treatment, has been denied by several workers but recently McIlarth and Ergle (1953) showed fairly conclusively that in cotton plant, the effect of 2,4-D persisted for a long time; it was located in the fruit; the typical 2,4-D effect was observed in the seedlings raised from these seeds; and finally a substance, identical with or a metabolite of 2,4-D, was isolated long after the treatment from distant untreated parts of the plant.

Finally, there does not seem to be any work carried out so far on the influence of time of sowing on the effect of 2,4-D on growth and maturity of wheat.

Methods and Material

Seeds of wheat C. 591 were kept in petri-dishes in contact with solutions of 10 and 100 ppm. of 2,4-D and with water for 24 hours at 24—26.5° C on 8th of Nov., 1954. A similar set was again kept on 4th of Dec., 1954 at 20—22° C. They were sown along with dry seeds (control) in pots on 9th Nov. 1954 and 5th Dec. 1954 respectively. Five pots were allotted to each treatment. Ten seeds were sown per pot and four weeks after sowing, the seedlings were thinned to three. The following observations were recorded: (i) sprouting during soaking; (ii) germination in pots; (iii) shoot-dry-weight (four weeks after sowing); (iv) height of the main shoot upto the base of the last open leaf or to the base of the ear, number of fully open

green leaves and number of tillers (including main shoot) at four weekly intervals upto maturity; (v) date of ear-emergence; (vi) dry weight of shoot (minus grain) at harvest; (vii) yield of grain; and (viii) 1,000 grain-weight.

All the results, except on 'sprouting percentage' (mean of duplicates), were statistically analysed by the analysis of variance method with five replications for 'germination percentage' (mean per pot) and for shoot-dry-weight at 4-week stage (mean per plant per pot), and 15 replications for the remaining observations.

The results for the two sowings were not combined in the statistical calculations, as, due to lack of suitable accomodation in the open, the November-sown pots had to be kept throughout in a glass-house, which was relatively warmer than the 'open' enclosure having the December-sown pots. Thus, while explaining the results, no attempt was made to compare the absolute values for the two sowings.

Results and Discussion

The detailed quantitative observations on germination growth and maturity are summarised in Table 1. The values of 'critical difference' at 5 per cent level are included for observations wherein the treatment-effect was significant.

Sprouting: 2,4-D affected adversely the sprouting of seeds during the 24 hour soaking period in November as well as in December; the effect was more pronounced in the higher concentration (100 ppm.). Sprouting of seeds was generally lower in December than in November.

Germination: Seeds soaked in water or in 10 ppm. of 2,4-D germinated as well as the dry seeds (control), when they were sown in November but the treatment, 100 ppm. of 2,4-D, resulted in only 64 per cent germination which was significantly lower than the other 'treatments'. In the December sowing, however, the differences in germination due to the 'treatments' were not significant, although 100 ppm. of 2,4-D gave 74 per cent germination compared to 86 per cent in the control set. Inspite of the relatively lower sprouting percentage during December than in November, germination was almost the same in both the sowings.

Growth: The effect of presowing soaking of wheat seeds for 24 hours in a solution of 2,4-D on growth of the plants varied with the concentration of the solution and also with the time of sowing.

10 ppm. of 2,4-D: The height of the November sown treated plants was significantly less than the control ('dry seed') till the plants were 8 weeks old; later on, the differences gradually disappeared and at maturity the treated plants were as tall as controls; number of green leaves and tillers were significantly lower than the control, only at the 8-week stage. In the December-sowing, however, there was an initial depression in the height of the 2,4-D treated plants at the 4-week stage; the treated plants recovered soon

Table 1. *Effect of 2,4-D on germination, growth, and maturity of wheat.* (Mean of 15 plants.)

Observations	November Sowing					December Sowing				
	Control ('dry seed')	Pre-sowing soaking in 2.4-D solution			C.D.[1]	Control ('dry seed')	Pre-sowing soaking in 2.4-D solution			C.D.[1]
		0-ppm.	10-ppm.	100-ppm.			0-ppm.	10-ppm.	100-ppm.	
Sprouting % (mean of duplicates)	—	64	53	34	—	—	47	39	27	—
Germination % (mean of 5 pots)	88	86	86	64	17	86	86	78	74	—
4-Week Stage:										
Height (cm.)	6.5	6.2	6.1	4.8	0.4	5.0	5.3	4.5	3.8	0.7
Leaves	2.9	2.9	2.9	2.4	0.3	3.2	3.4	3.5	2.5	0.5
Tillers	1.0	1.0	1.0	1.0	—	1.3	1.5	1.7	1.0	0.3
Shoot dry-wt. (mgm.)	55	61	58	35	9	82	89	65	30	10
8-Week stage:										
Height (cm.)	19.8	20.5	16.3	11.5	2.2	32.0	39.5	36.2	32.1	3.0
Leaves	6.5	6.0	5.4	5.2	0.7	12.7	15.1	18.5	11.4	1.8
Tillers	1.5	1.3	1.1	1.1	0.4	3.6	4.0	5.0	3.7	0.7
12-Week stage:										
Height (cm.)	67.0	67.3	63.3	50.9	6.7	—[2]	—	—	—	—
Leaves	7.3	7.3	7.5	6.3	—	—	—	—	—	—
Tillers	1.8	1.6	1.5	1.2	0.4	—	—	—	—	—
Ear-emergence (days)	92.0	90.2	90.9	98.4	2.5	80.9	79.4	80.0	81.7	1.2
Maturity stage:										
Height (cm.)	86.9	88.7	87.0	81.8	—	74.6	71.6	73.9	74.0	—
Leaves	7.2	5.9	6.7	6.8	—	9.6	9.2	10.1	7.3	1.4
Tillers	1.8	1.7	1.7	1.4	—	3.9	4.1	5.0	3.9	0.6
Shoot dry-wt. (gm.) ..	2.83	2.39	2.67	2.25	0.35	3.78	3.97	5.30	3.50	0.59
Grain (gm.)	1.28	1.29	1.50	1.21	0.28	1.62	1.82	2.16	1.45	0.39
1000 gr.wt. (gm.)	29.68	30.80	35.15	29.00	2.95	22.28	27.99	27.69	20.88	2.84

[1] C.D.: Critical difference at 5 % level.

[2] December-sown plants were almost mature in 12 weeks, thus observations for 12-week stage are included under Maturity stage.

and four weeks later they were even taller than the controls, although at maturity this stimulating effect disappeared; tillering was better than the control throughout while the number of green leaves was more than the control, only at the 8-week stage.

Thus, it can be said that the slight adverse effect of 10 ppm. of 2,4-D on growth (particularly height) persisted up to the 8-week stage in the November sowing, while, in the later sowing, it was restricted upto the 4-week stage, followed by a temporary stimulation. Moreover, in the later sowing the treated plants tillered better than the controls.

Table 2. *Effect of 2,4 D on dry weight of shoots and yield of grain in wheat C.591.* (Percentage on control.)

Treatment	November sowing 1954—55		December sowing			
			1954—55		1953—54[1]	
	Shoot	Grain	Shoot	Grain	Shoot	Grain
Control ('Dry seed')	100	100	100	100	100	100
2.4-D: 0-ppm.....	84	101	105	112	115	96
2.4-D: 10-ppm...	94	117	140	133	142	142
2.4-D: 100-ppm...	80	89	92	89	97	90
C.D. at 5 % level	12	22	16	24	13	16

[1] Results already reported by the authors (1955)

100 ppm of 2,4-D: In the November sowing, the adverse effect on growth was more pronounced with this concentration than with 10 ppm. and persisted longer i e. up to 12-week stage In the later sowing, the depressing effect of this concentration was confined to the 4-week stage as in the case of 10 ppm.; but the later temporary stimulatory effect, observed with the lower concentration of 2,4-D, was not seen with 100 ppm. In both the sowings, the treated plants recovered almost completely at maturity.

Few workers seem to have studied the effect of presowing soaking of seeds in 2.4-D solution on germination, growth and maturity of plants. Hamner, *et al.* (1946) observed lower germination and inhibition of top-growth of seedlings of wheat with 100,000 ppm. of 2,4-D, lower concentrations (10,000 ppm. and 1000 ppm did not inhibit germination or seedling growth but, as the presowing soaking period was only for four hours, their results are not strictly comparable with the present data. Stimulation of auxillary growth in bean plants treated with 1 or 10 ppm. of sodium salt of 2,4-D was noted by Stromme and Hamner 1948), it supports the better tillering of December-sown wheat plants treated with 10 ppm. of 2,4-D. Mitchell and Marth (1945), from their studies on the effect of spraying grasses with 2,4-D, observed — "in all cases (doses , the treatment caused an initial depression in the rate of growth. The depression gradually disappeared" — thus confirming the results of the present experiment.

Ear emergence· In November sowing, there was practically no difference in the date of earing between the control and treated sets except with 100 ppm. of 2,4-D where the plants eared 6.4 days later than the controls. In December-sowing the difference between the control and 2,4-D (both the doses) was negligible while water-soaking' resulted in a significant but slight earliness compared to 100 ppm. of 2,4-D.

Dry weight of shoot and yield of grain: For a better comparison, the values for the treatments are summarised as percentage on the respective controls
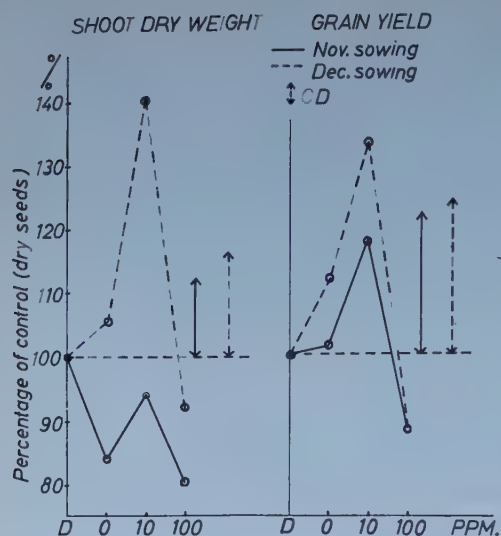
Figure 1. *Effect of 2,4-D on shoot-dry-weight at maturity and yield of grain of wheat C.591*; C.D. Critical difference at 5 % level.

(Table 2) and are also represented graphically (Figure 1). The results of 1953—54 experiment, already reported by the authors (1955), are also included in the table for comparison. The treatment effect is also clear as seen in Figure 2.

The salient feature in the above table is the stimulating effect of 10 ppm. of 2,4-D on shoot-dry-weight and yield of grain in December-sowing and its absence in the earlier sowing. Mitchell and Marth (1945) who mentioned about the gradual recovery from the initial depression in growth of grasses due to 2,4-D did not attach any importance to the apparently higher yield of clippings (525) at the end of 52 weeks with the lowest dose of 2,4-D ($^3/_4$ lb.), compared to 458 of control. Stromme and Hamner (1948) obtained an increase in the number of pods in bean plants treated with 1 or 10 ppm. of sodium salt of 2,4-D. Thus under certain conditions low concentrations of 2,4-D can increase growth and yield, as observed in the present study in wheat sown in December. It confirms the earlier results obtained by the authors (1955).

Quality of Grain: Even the 1000 grain-weight was higher with 10 ppm. of 2,4-D than in the respective controls in both the sowings; the values were 35.15 gms (2,4-D) and 29.68 (control) for the November sowing, and 27.69 gms and 22.28 gms respectively for the later sowing; the above differences in both the sowings were statistically significant. Again, the higher dose of 2,4-D gave apparently as good grain as the respective controls in the two sowings, because the small differences in the 1000-grain-weight were not significant. Water-soaking led to better quality of grain than the control,

Figure 2. *Effect of 2,4-D on growth of wheat C.591 sown in November* (left row) *and in December* (right row); photograph taken on 24-3-1955. — D: Control (dry seed); C: 0 ppm ('water soaking'); HL: 10 ppm.; HH: 100 ppm. of 2,4-D

but only in the later sowing. Rebstock *et al.* (1954) observed a doubling of the nucleic-acid in the stem of bean plants treated with 1000 ppm. which apparently caused the unusual growth. Thus 2.4-D seems to affect the P-metabolism which perhaps influences the quality of grain

Finally, the observation that the treatment given to wheat seeds for a short duration during germination influences the growth and yield, is supported by the conclusions of McIlarth and Ergle (1953) regarding the persistence of 2,4-D in remote regions of the plant. long after the treatment.

Summary

Seeds of wheat C. 591, after presowing soaking in 0, 10, and 100 ppm. of 2,4-D for 24 hours, were sown in pots along with control (dry seeds) in the first week of November and of December. Quantitative observations on germination, growth at different stages, yield and quality of grain were recorded. The results were analysed statistically.

The higher level of 2,4-D alone inhibited the germination of seeds in both the sowings.

An initial depressing effect on growth (particularly height) was noted for

the 2,4-D-treated plants in both the sowings The effect was more pronounced and persisted longer in the November sowing, especially with 100 ppm. of 2,4-D. In the later sowing, growth was temporarily stimulated with the 10 ppm. after the initial slight inhibition (4-week stage). December-sown plants of 100 ppm. recovered quicker than their counterparts of the earlier sowing. By maturity there was no significant difference in height due to the treatments in both the sowings.

In November sowing, the yield of dry-matter in shoots was significantly lower with 100 ppm. than the control. In the later sowing, 10 ppm. gave the highest value; 100 ppm. was as good as the control

The yield of grain in the earlier sowing was scarcely affected by 2,4-D (both the levels) when compared with the control. In the later sowing, however, 10 ppm. gave 33 per cent increase in grain over the control while 100 ppm. was almost as good as the control

In both the sowings, 1000 grain-weight was definitely better with 10 ppm. than the respective controls; even with 100 ppm., the quality of grain was equal to that of the controls.

The results support the earlier findings by the authors (1955).

References

Allard, R. W., DeRosa, H. R. & Swanson, C. P.: Some effects of plant growth-regulators on seed germination and seedling development. — Bot. Gaz 107. 575. 1946

Bhardwaj, S. N. & Rao, I. M.: Studies on the effect of 2,4-D, NAA and IAA on growth and maturity of wheat. — Jour. Ind. Bot. Soc. 34: 21. 1955.

Hamner, C. L., Moulton, J. E & Tukey, H. B.: Effect of treating soil and seeds with 2,4-D on germination and development of seedlings. — Bot. Gaz. 107. 352. 1946.

McIlarth, W. J. & Ergle, D. R.: Further evidence of persistence of the 2,4 D stimulation in cotton. — Plant Physiol. 28: 693. 1953.

Mitchell, J. W. & Marth, P. C.: Effects of 2,4-dichlorophenoxyacetic acid on the growth of grass plants. — Bot. Gaz. 107: 276. 1945.

Pande, H. K.. Effect of sodium dichlorophenoxyacetate on crop and weeds in wheat field — Agra Univ. J. Res. (Sci.) 3: 241. 1954.

Rebstock, T. L., Hamner, C. L. & Sell, H. M.: The influence of 2,4-D on the P-metabolism of Cranberry bean plants (Phaseolus vulgaris). — Plant Physiol. 29: 490. 1954.

Sen, P K. & Moolani, M. K.. Pre-sowing treatment of seeds with growth promoting substances. — Proc. Ind. Sci. Cong. (Pt. III), 255, 1954.

Stromme E R. & Hamner, C. L.: Delayed maturity of bean plants sprayed with solutions of 2,4-D of non-herbicidal concentrations. — Science 107: 170. 1948.

The Effect of 2,4-Dinitrophenol and Some Oxidase Inhibitors on the Oxygen Uptake in Different Parts of Wheat Roots

By

Lennart Eliasson and Inna Mathiesen

Botanical Laboratory, Lund
(Received March 14, 1956)

Introduction

The study of the physiological gradient along the longitudinal axis of roots has attracted some interest in connexion with the researches concerning growth phenomena. It has been shown that significant changes occur during growth in the protein content per cell (cf. Morgan and Reith 1954, Jensen 1955) and in the activity of various enzymes (Robinson and Brown 1952, 1954). The literature concerning the respiratory gradient has recently been reviewed by Betz (1955) and Eliasson (1955). The most characteristic feature of this gradient is that the gas exchange per unit protein nitrogen is greater during the cell extension than in the meristematic stage.

Betz, in an extensive investigation using roots of *Pisum sativum* and *Zea mays*, obtained about three times as high respiratory intensity in the extension zone as in the meristem. There are, however, reasons to suspect that the low values of oxygen uptake in the meristem obtained by Betz are, to some extent, due to oxygen deficiency, mainly because they are connected with unusual high R.Q. values. For Pisum roots Betz obtained the highest oxygen uptake in the differentiating zone (5—10 mm above the tip). Other workers have reported a gradual decline in the respiratory rate after the cessation of growth (cf. Eliasson). In an investigation with thin sections from the apical 3 mm of *Vicia faba* roots Jensen (1955), using ultramicromethods, found great changes in the metabolism already during the earliest growth stages. An investigation of the effect of glucose supply on oxygen uptake and R.Q. in excised segments of wheat roots (Karlsson and Eliasson 1955)

[265]

showed that the respiration in the part of the root where the extension growth has just been completed is less dependent on external substrate than in other parts.

Obviously there are important changes in the respiratory intensity during the different growth phases. Very little has however, been done in order to investigate the qualitative changes that may occur in the respiratory mechanism during growth. Investigations in this field are most desirable, constituting an important part of the study of the developmental processes in the root.

Even from another point of view, such investigations seem to be an urgent task. Whole root systems or a part thereof, mostly tips, have been a frequent object in studies of respiratory mechanisms. The conclusions drawn from such experiments are often highly dependent on the presumption that the material is homogeneous in respect to the investigated qualities. To what extent this is the case must, however, be closely investigated.

The purpose of the work recorded here has been to investigate the response to respiratory inhibitors of different parts of wheat roots. The inhibitors used have been 2,4-dinitrophenol (DNP), sodium azide, potassium cyanide, and sodium diethyldithiocarbamate (dieca).

Methods

Cultivation of the material. The experimental material was roots of wheat seedlings (Weibulls orig. "Eroica") cultured under sterile conditions using earlier described methods (see Eliasson 1955). The seeds were first germinated in sterile Petri dishes and then cultured for two days in sterile nutrient solution in the dark at $22°$ C. As the nutrient solution employed in the earlier published experiments (Eliasson 1955, Karlsson and Eliasson 1955) was found to have a depressing effect on the root growth, a solution with lower salt concentrations and higher initial pH (6.6) was used. The molar composition of this new solution was: 10^{-4} M Na_2HPO_4, 10^{-4} M KH_2PO_4, 10^{-4} M $Ca(NO_3)_2$, 5×10^{-5} M $MgSO_4$, 10^{-5} M $MnCl_2$, 10^{-6} M H_3BO_3, and 10^{-6} M Fe-citrat. The growth in length after two days in the new solution was 4.7 to 5.4 cm. In a series of experiments with seeds from the same source but with the old solution the growth varied from 3.1 to 4.3 cm.

Respiration determinations. The preparation of the roots and the measuring of the oxygen uptake was performed mainly according to the methods described by Eliasson. Only the three earliest developed roots were used. The lengths of the root segments employed were in order from the tip: 2 mm (mainly the meristematic zone, cf. Eliasson), 3 mm (the main part of the extension zone), 5 mm (the last part of the extension zone and the beginning of the differentiating zone). From the remainder of the root, pieces with a length of 10 mm were used. The oxygen uptake was determined at 22 C with standard Warburg technique. The shaking rate used was 125 oscillations a minute. The number of root pieces in each flask was varied according to their length and respiratory rate between 35 and 80, in such a way that the oxygen uptake in all the flasks was of approximately the same rate.

After the excision the root pieces were kept in contact with a 0.02 M phosphate buffer at pH 6.6 containing 0.05 M glucose. They were suspended in 2 or 2.5 ml of this solution in the Warburg flasks. The glucose was applied because the respiration is relatively constant in the presence of this substrate, whereas there is a decrease without substrate supply (see Karlsson and Eliasson 1955). It was found that a prerequisite of this technique is having the roots sterile at the beginning of the experiment. If the roots were cultured in non-sterile solutions, there were severe implications due to bacterial contaminations. This was especially true of the older parts of the roots after the roots had been kept for a few hours in glucose solution.

Application of inhibitors. DNP and azide were added from the sidearm. DNP was dissolved in buffer solution of the kind used for the roots. NaN$_3$ was dissolved in 0.001 M Na$_2$HPO$_4$ in order to diminish the concentration of the free acid HN$_3$ in the solution in the sidearm and hence the rate with which this distilled over to the solution in the main compartment of the flask. Cyanide dissolved in buffer solution was added directly to the experimental solution after removing the flasks from the manometers. At the same time the KOH in the center well was exchanged for a mixture of KCN+KOH, using the concentration given by Robbie (1948). In the experiments with the mentioned inhibitors, the oxygen uptake was determined for two hours before addition of the inhibitor. As the respiratory rate without inhibitors was, on the whole, constant throughout the experimental period the oxygen uptake during these two hours was used as the control value for the sample. Dieca was not added to the flasks as it is easily decomposed under gas evolution but the root segments were pretreated with a solution of the inhibitor by shaking for two hours as described by James and Garton (1952). The dieca solution was renewed every 20 minutes. Parallel samples treated in the same way with solution without dieca were used as the control in these experiments.

Oxygen diffusion as possible limiting factor. Especially for the meristematic zone, there is considerable danger that the rate of oxygen diffusion through the tissue may be the limiting factor for oxygen uptake when air is used as the gaseous phase (cf. Kandler 1950 a, Berry and Norris 1949). This possibility was investigated by determination of the oxygen uptake in atmospheres with 50 and 100 per cent oxygen. In the meristematic segment during the first two hours after the alteration of the oxygen tension, there was an increase in the oxygen uptake amounting to 10—25 per cent of the uptake in air. The other parts of the root showed no significant deviation from the oxygen uptake in air. The R Q was not affected by the higher oxygen tension in any part of the root. Thus the slow oxygen diffusion in the meristematic segment evidently has a slightly depressing effect on the oxygen uptake in air. Consequently, it was found that the stimulatory effect of DNP on the oxygen uptake in the meristem is somewhat greater in 100 per cent oxygen than in air. As for the rest, there is no reason to assume that this slight effect may have affected the results reported in this paper, in spite of the fact that the respiration determinations have been carried out in air. A fact which should make the use of higher oxygen tension proble-

matic in experiments like these is that oxygen has a reversing effect on the inhibition caused by azide (see table 1) and cyanide (cf. Lundegårdh 1954).

The respiratory gradient in relation to the age of the root. The divergences observed between different parts of the root have in this paper been interpreted as a result of the developmental and aging processes in the cells after their formation. This is, however, not the only possible interpretation. The properties of the meristem and the other zones of the root may change during the course of the root development, and there is a possibility that the divergences between root parts of different age partly are due to distinct properties already present at the time of cell formation in the meristem. This is especially indicated by the work of James and Boulter (1955). Using 10 mm long root tips of barley, they obtained respiratory inhibitions with several oxidase inhibitors which varied in degree with the age of the roots. To what extent such effects are implicated in the results reported here remains to be investigated. On the other hand, the results of James and Boulter may partly be due to an alteration of the length of the different physiological zones of the root tips, for instance by a change in the growth rate (cf. Eliasson 1955) which is likely to occur during the aging of the root.

Results

The effect of DNP. DNP is known to induce considerable disturbances in the cell metabolism, probably as a consequence of accelerated breakdown, combined with inhibited synthesis of energy-rich phosphate compounds like adenosinetriphosphate (ATP). This causes in most plant tissues a considerable stimulation of respiration at low concentrations and inhibition at higher. A thorough review of the literature up to 1950 is given by Kandler (1950 b). Later Beevers (1953) found that DNP induces aerobic fermentation in various plant tissues, and French and Beevers (1953) investigated and discussed the relation between the DNP effect and the respiratory stimulation caused by growth substances. In an investigation of the climacteric rise in fruit respiration Millerd et al. (1953) found that DNP stimulated the respiration before the climacteric rise but not during this or at the climacteric maximum.

The effect of DNP on the respiration of wheat roots has earlier been investigated by Stenlid (1949 b) who used 10—15 mm long tips of young seedlings. The aim of the present investigation has been to compare the effect of DNP on different parts of wheat roots. Two concentrations have been used, 5×10^{-5} M, giving maximal stimulation at the pH used, and 2×10^{-4} M, giving inhibition in the growing parts of the root. Figures 1 and 2 show the time courses of the oxygen uptake in different parts of the root after addition of DNP. Even if there is a considerable difference in the stimulation between the growing and the mature tissue, it appears from this and from other similar experiments that the most important divergence is in the sensitivity to the inhibitory effect of DNP.
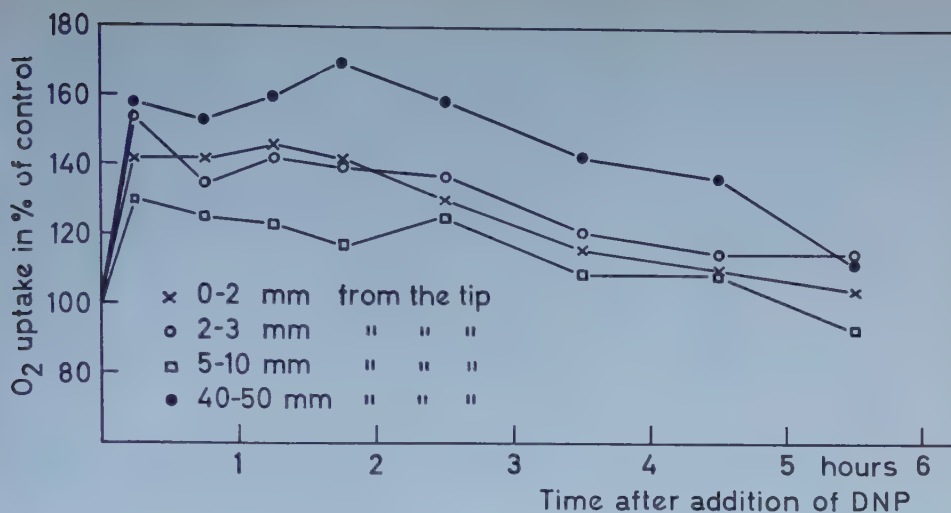
Figure 1. *The time course of the oxygen uptake in different parts of wheat roots in* 5×10^{-5} *M DNP*. The roots in 0.02 *M* phosphate buffer with 0.05 *M* glucose. The oxygen uptake before addition of DNP has been used as the control. DNP added from the sidearm. — Mean values of two parallel experiments.
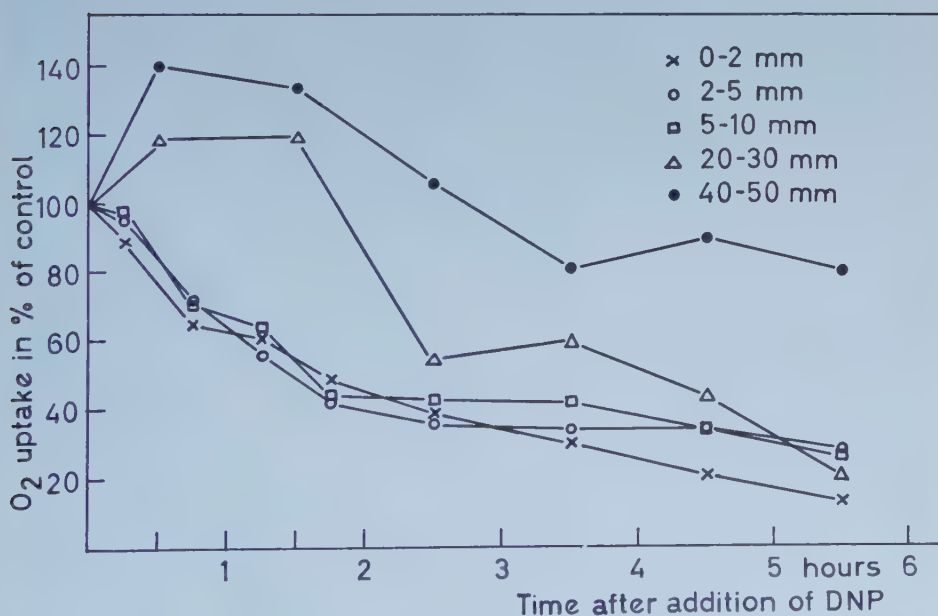
Figure 2. *The time course of the oxygen uptake in different parts of wheat roots in* 2×10^{-4} *M DNP*. For further explanations see figure 1.

Table 1. *The effect of azide on the respiration in different root segments.* The roots suspended in 2.5 ml 0.02 M phosphate buffer with 0.05 M glucose and 0.001 M NaN_3 at pH 6.6. Oxygen uptake in per cent of the control I: 0—2 hours, II and III: 2—4 hours after addition of azide. Column III with the air replaced by 100 per cent oxygen. — Column I mean values of five, column II of three, and column III of two determinations.

Distance from the tip mm.	O_2 uptake in per cent of control		
	I	II	III
0— 2	42	34	53
2— 5	55	49	62
5—10	64	53	60
30—40	78	61	95

As will be discussed in greater detail later, there is a close connexion between the stimulatory and the inhibitory effect of DNP, both probably being a result of a decrease in the concentration of ATP. An example of this connexion is the fact that the weak stimulation shown by the segment 5—10 mm from the tip is connected with a great sensitivity to the inhibitory effect of DNP. In contrast to this, the oldest parts of the root are characterized by a pronounced resistance against the inhibitory effect and a high stimulation over a relatively broad concentration range. Another point is that, while the stimulation in the mature root parts and even in the meristem is very reproducible, the segment 5—10 mm from the tip shows a rather variable stimulation from one experiment to another. Even in this segment it is sometimes possible to get a stimulation of about 50 per cent (see table 3). The meristematic segment shows some properties which distinguish it both from the juvenile but extended tissue in the segment 5—10 mm from the tip and from the mature tissue in the upper part of the root. As earlier pointed out, the low rate of oxygen diffusion has a depressing effect on the oxygen uptake in this segment, and this effect will be particularly pronounced at the high respiratory intensity caused by DNP. In fact, the maximal stimulation of the oxygen uptake in the tip segment in 100 per cent oxygen is of about the same order as in the mature tissue (unpublished experiments). On the other hand, there is a great sensitivity to the inhibitory effect of 2×10^{-4} M DNP (figure 2), which after three or four hours is even greater than in the other growing or juvenile tissues.

Azide and cyanide. The use of selective inhibitors has been thoroughly treated by James (1953 a). Azide and cyanide mainly inhibit iron and copper oxidases. Besides azide has an uncoupling effect on phosphorylation and may for this reason in certain concentrations have a stimulatory effect on the respiration in some tissues (Stenlid 1949 a, Harley and McCready 1953). This is, however, not the case for wheat roots (cf. Stenlid 1948 and 1949 b).

Table 2. *The respiration in different root segments in* 5×10^{-4} *M cyanide.* The roots suspended in 0.02 *M* phosphate buffer with 0.05 *M* glucose and cyanide. Center well with 0.3 ml 5 *M* KCN and 0.5 *M* KOH. Oxygen uptake in per cent of the control. Column I ${}^1/_2$—2 ${}^1/_2$ hours, column II 2 ${}^1/_2$—4 ${}^1/_2$ hours after addition of cyanide. — Mean values for three experiments.

Distance from the tip mm.	O₂ uptake in per cent of control	
	I	II
0— 2	30	27
2— 5	33	31
5—10	39	32
40—50	46	38

The values in table 1 show that the inhibition caused by azide is greatest in the meristem and decreases with the distance from the tip. In all segments there is an increase in the inhibition with time in air, but the inhibition is counteracted by 100 per cent oxygen, particularly in the meristematic and in the mature tissue. The azide concentration used in these experiments, 10^{-3} *M*, was chosen because it gave a respiratory inhibition of around 50 per cent at pH 6.6. The same concentration gives at lower pH almost complete inhibition of the oxygen uptake (cf. Stenlid 1948).

Table 2 shows that, in 5×10^{-4} *M* cyanide, there is a corresponding decrease in the respiratory inhibition with the distance from the tip. These results coincide with those of Lundegårdh (1949). He obtained in 10^{-3} *M* cyanide a decrease in the respiration of the wheat root to 35 per cent of the control in the 0—30 mm tip zone and to 56 per cent in the zone 30—60 mm from the tip. Even for Allium roots Berry and Brock (1946) found a greater sensitivity for cyanide in the 5 mm tip segment than in the subsequent 10 mm of the root. On the other hand, Wanner (1944) reported a greater cyanide inhibition in the "root hair zone" than in the apical parts of the wheat root.

In a concentration of 10^{-4} *M* cyanide the inhibition shows considerably less divergence between the different parts of the root (table 3). This coincides with the observation by Lundegårdh (1949) that the cyanide-sensitive respiration in the older parts of the root is more completely inhibited by 10^{-4} *M* HCN than in the 30 mm tip piece. In the latter zone there was a considerable further inhibition when the concentration was increased to 10^{-3} *M* HCN. The reversal of the inhibition shown in 10^{-4} *M* cyanide (table 3) also is of the same kind as that earlier found by Lundegårdh (1950, 1954). The most interesting point shown by the values in table 3 is that DNP does not stimulate the respiration in the presence of cyanide, while the same concentration of DNP gives about 50 per cent stimulation in normal roots. On the contrary, DNP prevents the reversal of the oxidative processes.

Table 3. *The effect of cyanide in low concentration and DNP on the respiration.* The roots in 0.02 M phosphate buffer with 0.05 M glucose at pH 6.6. In flasks with cyanide the KOH in the center well is replaced with 1 M KCN+0.5 M KOH. Oxygen uptake in per cent of the uptake during two hours before addition of cyanide. DNP added after respiratory measurements for another two hours.

Distance from the tip mm.	KCN added (M)	O_2 uptake in per cent Time in hours after addition of cyanide				
		$\frac{1}{2}$—$1\frac{1}{2}$	$1\frac{1}{2}$—$2\frac{1}{2}$	DNP added (M)	3—4	4—5
0— 2	10^{-4}	48	62	—	85	104
	10^{-4}	50	59	5×10^{-5}	71	58
	—	97	97	5×10^{-5}	151	146
	—	97	95	—	104	102
2— 5	10^{-4}	50	73	—	94	115
	10^{-4}	55	68	5×10^{-5}	72	80
	—	100	102	5×10^{-5}	155	155
	—	106	103	—	99	100
5—10	10^{-4}	53	63	—	71	84
	10^{-4}	61	67	5×10^{-5}	68	68
	—	99	106	5×10^{-5}	156	143
	—	103	95	—	101	97
40—50	10^{-4}	62	67	—	93	109
	10^{-4}	68	71	5×10^{-5}	84	93
	—	105	100	5×10^{-5}	165	160
	—	110	104	—	106	105

This shows that cyanide causes a great modification of the respiration even when the inhibition is slight. The phosphorylative processes are no longer limiting for the respiration. Stenlid (1949 b) showed that the azide has the same inhibitory effect on the power of the roots to react on DNP treatment with a respiratory stimulation.

Dieca. According to James and Garton (1952), dieca may be regarded as one of the most suitable selective inhibitors of copper oxidases. The methods used for pretreatment of the tissue in the experiments reported here have been worked out by James and Garton (1952) and extensively used on barley root tips (James 1953 b, James and Boulter 1955). These workers are of the opinion that dieca gives a good differentiation between the oxygen uptake mediated by cytochrome oxidase and that mediated by ascorbic acid oxidase in barley roots.

After pretreatment for two hours with 2×10^{-4} M dieca at pH 5, the different parts of wheat roots showed the following oxygen uptake in per cent of the control values:

            0— 2 mm 80 per cent
            2— 5 mm 65 ,,   ,,
            5—10 mm 58 ,,   ,,
          30—40 mm 80 ,,   ,,
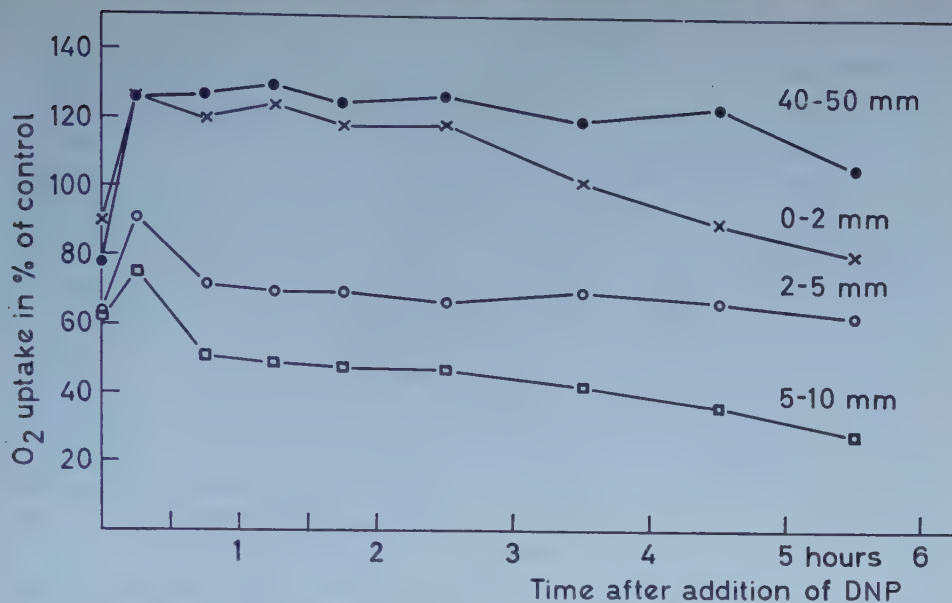
Figure 3. *The time course of the oxygen uptake of roots pretreated with 2×10^{-4} M dieca after addition of 5×10^{-5} M DNP.* The oxygen uptake in parallel samples not treated with dieca or DNP has been used as the control. For further explanations see figure 1.

The figures are the mean of seven determinations. The respiration of the inhibited roots was constant for at least six hours after the beginning of the experiment, in spite of the fact that the inhibitor was not present in the solution during the determinations.

If DNP is added in stimulatory concentrations to roots pretreated with dieca, there will be a great difference in the effect of DNP on the slightly inhibited tip segment and mature tissue, on the one hand, and the more strongly inhibited extending and newly extended tissue on the other hand (figure 3). Evidently there is a much more profound effect of dieca on the latter than on the former part of the root. A comparison with the effect of cyanide on the power of the tissue to react with a stimulation on DNP treatment (table 3) shows further that there are fundamental differences in the nature of the inhibition caused by dieca and that caused by canide. The respiratory system which is most important in generating high energy phosphates in meristematic and mature tissue is evidently not affected by dieca.

Discussion

The mechanism of DNP action. The problem of the action of DNP and its relation to oxidative phosphorylation in mitochondrial systems has been

excellently reviewed by Hunter (1951). More recent reviews are given by Anfinsen and Kielley (1954) and Green and Beinert (1955).

The most well-established primary effect of DNP is a strong stimulation of the adenosinetriphosphatase activity of the mitochondria. Besides DNP probably disturbs the power of the mitochondria to carry out phosphorylations coupled to electron transfer. The formation of energy-rich phosphate bonds on the substrate level is, on the other hand, evidently not disturbed by DNP (cf. Anfinsen and Kielley 1954). The increased supply of phosphate acceptors, such as adenosinediphosphate (ADP), will lead to a respiratory increase provided that the phosphorylatory processes are the limiting factor for respiration, which is usually the case in most tissues.

It may be stressed that the respiratory enzymes present in a tissue normally do not function at full efficiency. To obtain the true oxidative capacity of a tissue it is necessary to work under such conditions that "the rate of ATP breakdown is closely balanced with that of ATP synthesis" (Glass 1951, p. 699). If the breakdown of ATP is too slow, the respiration is slowed down by lack of phosphate acceptors. If, on the other hand, the ATP breakdown is too rapid, this will also give a decrease in the respiration. The oxidative capacity of the tissue is evidently dependent on a certain concentration of ATP to be able to function (cf. Potter and Rechnagel 1951).

Thus both the stimulatory and the inhibitory effect of DNP may be explained as the result of a single primary action, viz. a lowering of the ATP concentration. This primary action is presumed not to affect directly the structure of the mitochondria (cf. Anfinsen and Kielley 1954). As a result of DNP treatment, changes in the permeability of the cell membranes and increased exudation from the cells have often been observed, and it has been suggested that the DNP inhibition is due to some influence on the structural organization of the cell (cf. Shacter 1955). There are, however, no reasons to assume that this is a primary action of DNP. Damages to the structure of the cell may be explained as secondary effects due to the low ATP concentration.

The extent to which it is possible to stimulate the respiration with DNP varies from one tissue to another. In general, the stimulation is great in mature tissue and low in growing tissue (cf. Beevers 1953). French and Beevers (1953) obtained 70—80 per cent stimulation of the oxygen uptake in old Zea coleoptiles but only 30 per cent stimulation in growing coleoptiles. Kandler (1950 b), however, reported that it was possible to obtain a rise in the respiration of 20 mm long Zea root tips with about 300 per cent through long exposure to a low concentration of DNP. From the results in this paper, it appears as if the divergence between mature and growing root tissue is mainly a difference in the sensitivity to the inhibitory effect of DNP. In accordance with the above-discussed interpretation of the mechanism of DNP action, this implies that the balance between the ATP synthesized and

that broken down will more easily become negative in growing tissue than in mature.

DNP and the effect of supplied substrate. As has been shown in a previous communication (Karlsson and Eliasson 1955), there is a decrease in the respiratory rate of wheat roots not supplied with external substrate after excision. This well-known phenomenon was, as is usually done (cf. James 1953 c, Anker 1953), interpreted as due to lack of respirable material in the roots. This interpretation seems, however, difficult to reconcile with the picture of DNP action related above. DNP gives about the same rise in the respiration of starving roots as in the presence of external substrate (cf. Stenlid 1949 b). Thus it ought to be the breakdown of ATP and not the availiility of respiratory substrates that is limiting for the respiration even in this "starving" tissue. The observed decrease in the respiratory rate may be a consequence of lack of material for ATP-consuming synthetizing processes. This conception somewhat complicates the interpretation of respiratory drifts and feeding experiments. Probably there is some connexion between the high sensitivity to the inhibitory effect of DNP in the segment 5—10 mm from the tip and the comparatively small effect of glucose on the same part of the root reported by Karlsson and Eliasson.

The oxidase inhibitors. Although there have been many statements that the cytochrome oxidase disappears during the early development of the wheat seedlings (see Waygood 1950), it may now be considered as established that the cytochrome system functions even in roots from older wheat plants (Lundegårdh 1954, cf. also Hill and Hartree 1953). Besides there are good evidences for the functioning of an ascorbic acid oxidase system in wheat (Waygood 1950) as in barley (James 1953 b) and pea (Mapson and Moustafa 1956). Both these oxidases are inhibited by cyanide and azide. The fact that these inhibitors depress the respiration to a lesser degree in the older parts of the root than in the younger ones, indicates that there will be changes in the respiratory mechanism during the aging of the tissue. It is, however, hardly possible to decide the nature of these changes from the results reported here. The most immediate explanation would perhaps be that the two oxidases decrease in activity as the tissue grows older. However, there are some complications which must be considered. One of these is that there are some evidences for the existence of modifications of the cytochrome oxidase which are insensitive to cyanide (Thimann et al. 1954, Lundegårdh 1954). Thus an alternative explanation of the observed differences would be that modifications of the cytochrome system occur during the aging of the root cells so that it becomes less sensitive to cyanide.

Another complication is connected with the fact that the oxidases in the tissue normally do not function at maximal efficiency. For this reason it is incorrect to

assume that the decrease in oxygen uptake caused by a selective oxidase inhibitor represents the whole portion of oxidation mediated through that oxidase in non-inhibited tissue, as the inhibited enzyme might have had a sufficient activity to mediate a much greater oxygen uptake. Furthermore, if cytochrome oxidase is inhibited, there are at least two mechanisms which may be assumed to increase the efficiences of the oxidases not inhibited. 1. A greater fraction of the electron carrier system will come into the reduced state (cf. Lundegårdh 1955). This will increase the fraction of the non-inhibited terminal oxidases that is reduced and their rate of reduction and thus the rate of oxygen uptake via them. 2. The balance ATP synthesized—ATP broken down will be altered in such a way that the supply of phosphate acceptors will not be the limiting factor for the oxygen uptake. This is illustrated by the values in table 3. Thus it is probable that the oxygenating enzymes, functioning in cyanide-inhibited tissue, in the non-inhibited tissue mediate a much lower oxygen uptake. A third possible explanation of the difference in the cyanide-resistant oxygen uptake between young and old tissue might thus be the development of a normally more or less "latent" oxidase system, capable of taking over the function of the oxidase systems inhibited by azide and cyanide.

The experiments with dieca, on the other hand, provide good evidences that there are some changes in the role played by a copper oxidase, probably ascorbic acid oxidase, during the different developmental stages of the root. The experiments where DNP is added to dieca-treated roots (figure 3) reveal that the real difference in dieca inhibition between the various root segments is much greater than the values of the inhibition obtained in the usual way suggest. Before the addition of DNP the availibility of phosphate acceptors is the limiting factor for the respiratory processes in the meristem and in the mature tissue. For that reason the oxygen uptake in presence of DNP gives a better idea of the part of the oxidative mechanism that is not affected by the dieca inhibition. The reservation should, however, be made that dieca may have increased the sensitivity to the inhibitory effect of DNP especially in the segment 5—10 mm from the tip, and that this may account for a part of the difference in the oxygen uptake in presence of DNP.

Ascorbic acid oxidase is generally assumed to be a soluble enzyme (Goddard and Stafford 1954, Mapson and Moustafa 1956). In this connexion it is, however, of particular interest that Newcomb (1951) has found the enzyme associated with the cell wall fraction of tobacco stem pith cultures. He also found that the ascorbic acid oxidase activity increased as a result of auxin-induced growth. These results strongly suggest that the marked dieca inhibition in the extending and juvenile tissue affect ascorbic acid oxidase, formed during the cell extension and associated with the cytoplasm in contact with or interpenetrating the cell wall. Further, after the completion of the cell wall growth, when a more stabile cytoplasmic boundary layer is formed, this enzyme is evidently inactivated. The "extra" oxygen uptake

mediated by the oxidase may partly be responsible for the high respiratory rate per unit protein nitrogen found in the extension zone.

An aspect of the inhibitory effect of DNP may be mentioned in this connexion. As has been discussed above, this effect is probably due to a decrease of the concentration of ATP, which damages the structural organization of the cell. It may be assumed that processes dependent on soluble enzymes are not so sensitive to an inhibition of this type as the mitochondrial processes. The fact that the glycolysis is inhibited only at a higher DNP concentration than the oxygen uptake (Beevers 1953) supports this assumption. The question may now be raised whether the dieca-inhibited oxygen uptake is equally sensitive to the inhibitory effect of DNP as is the remainder of the respiration. This matter has not yet been investigated. However, certain results indicate that there is a fraction of the respiration of the part of the root 2—10 mm from the tip that is not so easily inhibited by DNP as the corresponding fraction of the meristematic tissue (cf. figure 3).

Summary

The effect of 2,4-dinitrophenol, azide, cyanide, and diethyldithiocarbamate on the oxygen uptake of different excised growth zones and mature parts of about five cm long, rapidly growing wheat roots has been investigated.

It is found that there is a greater sensitivity to the inhibitory effect of dinitrophenol in the growing and juvenile parts of the root than in the mature parts. Especially in the extending and newly extended tissue, this great sensitivity is connected with a lower maximal stimulation by dinitrophenol than in the mature tissue. Both the stimulatory and the inhibitory effect of dinitrophenol are interpreated as a result of a single primary action, affecting the concentration of adenosinetriphosphate.

The sensitivity to azide and cyanide inhibition is greatest in the meristem and decreases with the distance from the tip. Possible interpretations of this fact are discussed.

The inhibition by dieca shows a maximum in the root part 5—10 mm from the tip, about the end of the extension zone. The possibility that this is connected with an activation of ascorbic acid oxidase during the extension growth is discussed.

References

Anfinsen, C. B. & Kielley, W. W.: Biological oxidations. — Ann. Rev. Biochem. 23: 17. 1954.

Anker, L.: The effect of indole acetic acid and other growth promoting substances on the endogenous respiration of the Avena coleoptile. — Acta Bot. Neerl. 2: 22. 1953.

Beevers, H.: 2,4-dinitrophenol and plant respiration. — Am. J. Bot. 40: 91. 1953.

Berry, L. J. & Brock, M. J.: Polar distribution of respiratory rate in the onion root tip. — Plant Physiol. 21: 542. 1946.

— & Norris, W. E. Jr.: Studies on onion root respiration. I. — Velocity of oxygen consumption in different segments of root at different temperatures as a function of partial pressure of oxygen. — Biochem. Biophys. Acta 3: 593. 1949.

Betz, A.: Zur Atmung wachsender Wurzelspitsen. — Planta 46: 381. 1955.

Eliasson, L.: The connection between the respiratory gradient and the growth rate in wheat roots. — Physiol. Plant. 8: 374. 1955.

French, R. C. & Beevers, H.: Respiratory and growth responses induced by growth regulators and allied compounds. — Am. J. Bot. 40: 660. 1953.

Glass, B. in Phosphorus Metabolism 1: 658. Baltimore 1951.

Goddard, D. R. & Stafford, H. A.: Localization of enzymes in the cells of higher plants. — Ann. Rev. Plant Physiol. 5: 115. 1954.

Green, D. E. & Beinert, H.: Biological oxidations. — Ann. Rev. Biochem. 24: 1. 1955.

Harley, J. L. & McCready, C. C.: A note on the effect of sodium azide upon the respiration of beech mycorrhizas. — New Phytol. 52: 83. 1953.

Hill, R. & Hartree, E. F.: Hematin compounds in plants. — Ann. Rev. Plant Physiol. 4: 115. 1953.

Hunter, F. E.: Oxidative phosphorylation during electron transport. — Phosphorus Metabolism. McElroy and Glass, eds. 1: 297. Baltimore 1951.

James, W. O.: The use of respiratory inhibitors. — Ann. Rev. Plant Physiol. 4: 59. 1953 a.

— The terminal oxidases in the respiration of the embryos and young roots of barley. — Proc. Roy. Soc. B 141: 289. 1953 b.

— Plant Respiration. — Oxford 1953 c.

— & Boulter, D.: Further studies of the terminal oxidases in the embryos and young roots of barley. — New Phytol. 54: 1. 1955.

— & Garton, N.: The use of sodium dietyldithiocarbamate as a respiratory inhibitor. — J. Exp. Bot. 3: 310. 1952.

Jensen, W. A.: A morphological and biochemical analysis of the early phases of cellular growth in the root tip of Vicia faba. — Exp. Cell Res. 8: 506. 1955.

Kandler, O.: Untersuchungen über den Zusammenhang zwischen Atmungsstoffwechsel und Wachstumsvorgängen bei in vitro kultivierten Maiswurzeln. — Z. Naturforschung 5 b: 203. 1950 a.

— Über den Einfluss von 2,4-Dinitrophenol auf Atmung und Wachstum in vitro kultivierten Maiswurzeln. — Z. Naturforschung 5 b: 338. 1950 b.

Karlsson, B. & Eliasson, L.: The respiratory quotient in different parts of wheat roots in relation to growth. — Physiol. Plant. 8: 561. 1955.

Lundegårdh, H.: Quantitative relations between respiration and salt absorption. — Ann. Roy. Agr. Coll. Sw. 16: 372. 1949.

— Reversal of inhibition of respiration and salt absorption by cyanide and azide. — Nature 165: 513. 1950.

— Enzyme systems conducting the aerobic respiration of roots of wheat and rye. — Ark. f. Kemi 7: 451. 1954.

— On partial oxidation of the cytochrome system in the presence of cyanide. — Physiol. Plant. 8: 95. 1955.

Mapson, L. W. & Moustafa, E. M.: Ascorbic acid and glutathione as respiratory carriers in the respiration of pea seedlings. — Biochem. J. 62: 248. 1956.

Millerd, A., Bonner, J. & Biale, J. B.: The climacteric rise in fruit respiration as controlled by phosphorylative coupling. — Plant Physiol. 28: 521. 1953.

Morgan, C. & Reith, W. S.: The compositions and quantitative relations of protein and related fractions in developing root cells. — J. Exp. Bot. 5: 119. 1954.

Newcomb, E. H.: Effect of auxin on ascorbic oxidase activity in tobacco pith cells. — Proc. Soc. Exp. Biol. & Med. 76: 504. 1951.

Potter, V. R. & Recknagel, R. O.: The regulation of the rate of oxidation in rat liver mitochondria. — Phosphorus Metabolism. McElroy and Glass, eds. 1: 377. Baltimore 1951.

Robbie, W. A.: Use of cyanide in tissue respiration studies. — Methods in Medical Research 1: 307. Chicago 1948.

Robinson, E. & Brown, R.: The development of the enzyme complement in growing root cells. — J. Exp. Bot. 3: 356. 1952.

—   —   Enzyme changes in relation to cell growth in excised root tissues. — J. Exp. Bot. 5: 71. 1954.

Shacter, B.: Interrelations in respiratory, phosphorylative and mitotic activities of Ehrlich ascites tumor cells: influence of dinitrophenol. — Arch. Bioch. Bioph. 57: 387. 1955.

Stenlid, G.: The effect of sodium azide on the exudation and oxygen consumption of excised plant roots. — Physiol. Plant. 1: 185. 1948.

—   Some notes on the effect of sodium azide, 2,4-dinitrophenol, and ortho-phenanthroline upon oxygen consumption in green leaves. — Physiol. Plant. 2: 61. 1949 a.

—   The effect of 2,4-dinitrophenol upon oxygen consumption and glucose uptake in young wheat roots. — Physiol. Plant. 2: 350. 1949 b.

Thimann, K. V., Yocum, C. S., & Hackett, D. P.: Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. — Arch. Bioch. Bioph. 53: 239. 1954.

Wanner, H.: The zonal graduation of respiratory intensity in the root. — Ark. f. Bot. 31 A: 9. 1944.

Waygood, E. R.: Physiological and biochemical studies in plant metabolism. II. Respiratory enzymes in wheat. — Canad. J. Res. C 28: 7. 1950.

Light-Induced Stomatal Movements in Wheat Leaves Recorded as Transpiration

Experiments with the Corona-Hygrometer

By

Hemming I. Virgin

Botanical Laboratory, Lund
(Received March 16, 1956)

Introduction

The mechanism by which the stomata change their aperture has been the subject of a great number of investigations. It can now be considered well established that the active stomatal movements are based on turgor changes caused by alterations in the osmotic values of the guard cells. The resulting changes in the diffusion pressure deficit cause water movements and consequently changes in the shape of the guard cells according to their anatomical construction. A response in this way can be produced by altering many different factors. Among these light, water content, pH, and temperature are the most important ones, but shock stimuli, changes in ionic composition of the medium and other interventions can also affect the aperture; for reviews, see Heath (11, 12), Hygen (14), Scarth and Shaw (20), Stålfelt (28), Williams (31, 32). A response to so many different kinds of stimuli indicates that the aperture is not regulated by a single mechanism alone but by a combination of several. A support for this assumption is the fact that also light absorbed by other pigments than the chlorophylls seems to be active in the light response (Harms, 10; Liebig, 15; Paetz, 16; Sierp, 21).

The present investigation is a study of the light-induced stomatal movements recorded as transpiration. Due to improved instrumental equipment, determination of the different phases in the stomatal movement can now be studied with a great degree of accuracy. The paper deals mainly with pro-

[280]

cesses occurring in connection with changes from darkness to light and *vice versa* and with the behaviour of the stomata in intermittent light. For the illumination white incandescent light was employed.

Material and Methods

The plant material used for the experiments consisted of 10-day-old seedlings of wheat (Weibull's orig. "Eroica"). The seeds were soaked for 12 hours in tap water and germinated in Petri dishes on wet filter paper at 25° C. They were transferred after 2 days to a nutrient solution according to Rufelt (19). During the further growth the seedlings were kept under constant illumination — fluorescent lamps, daylight type — at the same temperature. The cultivation vessels were continuously aerated. The seedlings thus obtained had an approximate length of 10 cm.

The apparatus for the determination of the transpiration — the corona hygrometer — used in this investigation has been described in two earlier papers (Andersson and Hertz, 2; Andersson *et al.*, 3). It is now possible, due to improvements, to register changes in the humidity of the air as small as 0.5 per cent. This will allow very accurate measurements of even comparatively small changes in the transpiration, *i.e.*, stomatal aperture. The greatest possible sensitivity of the instrument will be obtained if the angle of the very tip of the platinum wire, serving as the anode, is about 90°.

In the beginning of the experiments some disturbances occurred frequently in the electrical discharge due to dust particles which collected on the platinum wire. In spite of thorough cleaning of the streaming air, this happened now and then and was very annoying. As most of the dust particles are negatively charged, the incoming air must, therefore, be extremely clean as all particles will be attracted to the anode. A way of avoiding this trouble is to let the air pass through a grounded brasstube, 20 cm in length, with an inner diameter of 1 cm, inside of which a stiff brass wire is placed, positively charged with the same voltage as the anode in the discharge chamber. In this way all dust particles are caught before they reach the anode. After applying this improvement no disturbances have taken place, and the apparatus can be used day after day without adjustments. It is very important, however, that the air-cleaning device be put under high tension before the air stream is allowed to pass the chamber.

For calibrating purposes different values for the humidity of the incoming air were obtained by letting it pass through glass tubes, 1 m in length, half filled with sulphuric acid of proper concentrations. It is important not to let the air bubble through the solutions, as in such cases microscopic droplets will be formed which sooner or later find their way to the platinum wire and will jeopardize the measurements.

For the illumination incandescent light from a projection lamp (Philips, type 297 G, 220 volts, 1000 watts) was used. The light passed through a water layer, 10 cm thick, which absorbed most of the heat radiation before it reached the chamber with the plants. The intensity of the light was measured by means of a Weston photocell.

The chamber for the illumination experiments was built of "Perspex" glass with the inner dimensions $120 \times 40 \times 4$ mm. The chamber was open at one of the short ends where the seedlings were inserted. The space around their bases was filled by modelling clay. Ten seedlings were used at a time. The leaves were placed so that they did not cover each other. The air entered the chamber through two "Perspex" tubes, fastened at the two sides of the chamber near the open end. From these side tubes the air entered the chamber through 6 small channels on each side of the chamber. At the upper end the air left the chamber through a perforated lamella in connection with the air-collecting tube, from which the air stream was led to the discharge chamber. In this way was obtained the greatest possible homogeneity of the air stream during its passage through the chamber. Check measurements with smoke as an indicator of the air turbulence, with as well as without seedlings in the chamber, did not show any special pathway for the air.

During the experiments the seedlings were intact with their roots immersed in aerated nutrient solution. In this way the plants were treated under as "normal" conditions as possible. The speed of the air in the chamber amounted in most experiments to 4 m per minute giving nearly maximal transpiration at any stomatal aperture (Stålfelt, 25). The temperature was kept constant at $22°$ C.

In the experiments reported in this paper the object was to study the photophase of the stomatal movement. For this reason the hydroactive closing mechanism should be eliminated to the greatest possible degree. This means that the water content of the leaves had to be kept at a high level. Guttation always occurred within one minute after the light had been switched off — in spite of a continuous stream of air. In some cases guttation droplets also appeared during illumination. This means that the water deficit in the plant as a whole seldom reached any high level. The possibility, however, that the mesophyll layers, being in contact with the stomata and situated between the conductive tissue, may have varied in respect to water level can not be excluded (Williams, 33). Actual measurements of the water deficit were not performed. The appearance of small guttation droplets on the tip of the leaves did not change the humidity of the air. The transpiration from the surfaces of the droplets is thus negligible in comparison to the cuticular and stomatal transpiration from the plants.

According to Gregory et al. (8) a change in leaf water content over the range of 70 to 100 per cent of the water present in turgid state has no significant effect in determining the rate of water loss from leaves of wheat and Pelargonium. This finding, however, does not agree with the experiments by Stålfelt (24, 28) who found that the stomata close at a water deficit exceeding 3 to 4 per cent — even under high intensities of light. The close relationship between stomatal aperture and water content has also been confirmed by several authors using a number of different plants. (de Alvim, 1; Pisek and Winkler, 17; Williams, 33; Williams and Barrett, 34; Yemm and Willis, 35).
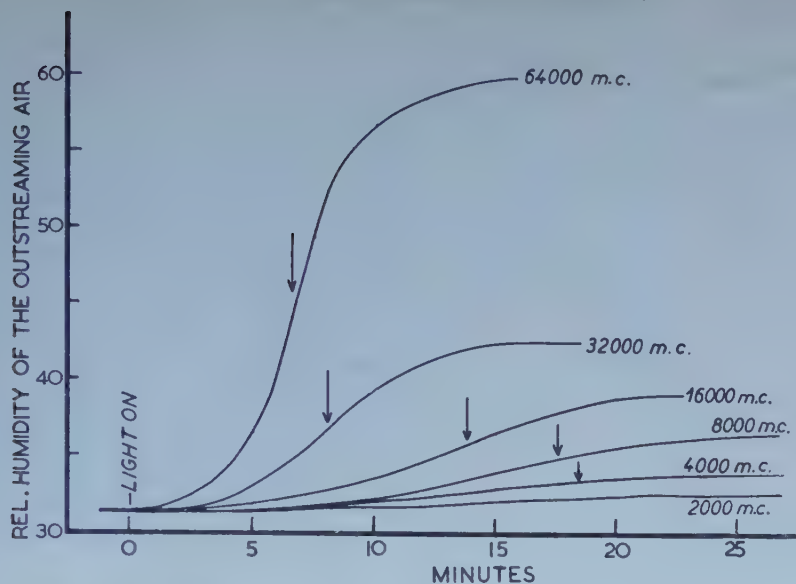
Figure 1. *The transpiration from wheat leaves under different light intensities.* Before illumination the leaves had been in darkness for 30 minutes. Transpiration value in darkness 32 %. Humidity of the incoming air 30 %. The arrows indicate the time when the rate of increase in the transpiration is the greatest.

Transpiration and Stomatal Aperture

The rate of stomatal transpiration is closely related to the aperture of the stomata. Stålfelt (25, 26, 27) found in studies of Avena and Betula that, except for the very first stages in the opening process where the increase in transpiration is very rapid, the relationship is almost linear, provided the wind velocity exceeds a certain minimum value. The explanation for the proportionally higher transpiration values at very small apertures as compared to the transpiration at larger was given by Bange (4). He showed that due to the shape of the stomatal opening the transpiration curve is to be considered as a combination of two components, each of them linear. One consists of the transpiration through a very narrow slit; the other of the transpiration through a broader slit. As the stomatal channel, leading to the intercellular spaces underneath the guard cells, often has the shape of an hour glass, these two components will always be in force.

The change in stomatal aperture with time can be calculated from the relationships transpiration *vs* time on the one hand and transpiration *vs* stomatal aperture on the other. There is no detailed information in the literature as to the transpiration *vs* stomatal aperture for wheat leaves, but there
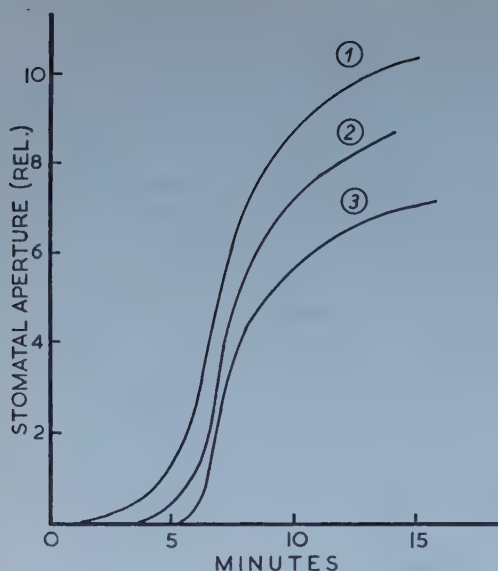
Figure 2. *The change in stomatal aperture by time after onset of illumination (64,000 meter candles).* The curves are calculated from values given by Stålfelt (27, Fig. 3) combined with values from Figure 1 (64,000 meter candles) in the present paper. Curve No. 1 represents values obtained presuming a high cuticular transpiration. Curve No. 3 is obtained calculating with no cuticular transpiration. Curve No. 2 gives the probable course of the stomatal movement with a cuticular transpiration corresponding to values found in the present experiments.

is no reason why this relationship should differ considerably from that found be valid for oat by Stålfelt (27). The shape of the stomata of these plants do not differ to any great extent. The transpiration *vs* time during the opening phase of the stomata is shown in Figure 1 for different light intensities. If the values in this experiments (the curve for 64,000 meter candles) are combined with the values given by Stålfelt (27, Fig. 3) for transpiration *vs* stomatal aperture, curves are obtained as shown in Figure 2. The effect of different values for the cuticular transpiration will be obtained by changing the initial level for the transpiration. In the Figure three different possibilities are given. Even if the deviations from the two "standard" curves may vary considerably from case to case, depending on varying experimental factors, the resultant curve for stomatal aperture *vs* time will have a sigmoid shape essentially similar to the curve for the transpiration *vs* time. The course of the transpiration shown in the experiments reported in this paper can therefore be considered to give a fairly true picture of the course of the change in stomatal apertures. As far as transpiration *vs* aperture is concerned the deviations from the straight line are greatest at small apertures. The greatest variations in the calculated curves will therefore be found during the first phases of the opening movement.

The general course of the transpiration from a wheat leaf in darkness and light as measured with the corona-hygrometer has been shown by Rufelt (19). The visible effect of light comes after a varying period of time — expansion phase — ("Spannungsphase"; Stålfelt, 23) during which no change in the

Figure 3. *The relation between the period in darkness before illumination and stomatal response.* The Figure shows the time required after the onset of illumination to reach a certain rate of transpiration. In this case 50 % of relative humidity is chosen as standard transpiration value. The same kind of curve is obtained if only the extension phase is measured. It is difficult, however, to determine exactly when the stomata begin to open. Light intensity was 64,000 meter candles.
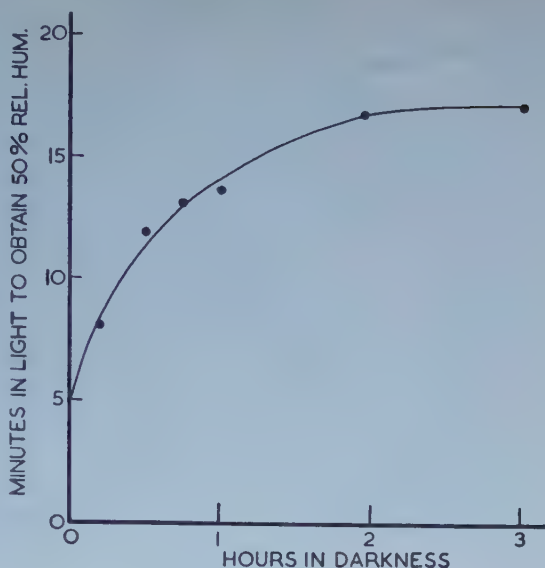
aperture of the stomata can be shown. The length of this period depends on several factors, among which pretreatment seems to play a great rôle. The variation in length of the expansion phase after different periods of darkness in these experiments is shown in Figure 3.

The opening phase ("motorische Phase"; Stålfelt, 23), i.e., the change in the transpiration rate during the first 10 to 20 minutes of illumination due to an increase in stomatal aperture, follows a sigmoid curve. This indicates that the speed at which the stomata open is first rather slow, increases and thereafter slows down again until the maximum aperture is reached under the actual conditions. The changes in transpiration during this opening phase depend on the light intensity (Figure 1), whereby the product law is in force with certain limitations (Stålfelt, 23; Harms, 10). See also Figures 7 and 8. In Figure 1 it can be seen that the maximum rate of change in aperture will be reached the earlier the higher light intensity.

Plants which have been in darkness for several hours do not show any immediate response in respect to transpiration when illuminated (Figure 4). This proves that the cuticular transpiration from wheat leaves does not change with increased intensity of illumination. The cuticular transpiration is also low in comparison to the maximal transpiration when the stomata are wide open.

The absolute magnitude of the cuticular and stomatal transpiration respectively can be calculated from the values obtained for the humidity of the air which has passed over the plants. In most of the experiments the incoming air had a relative
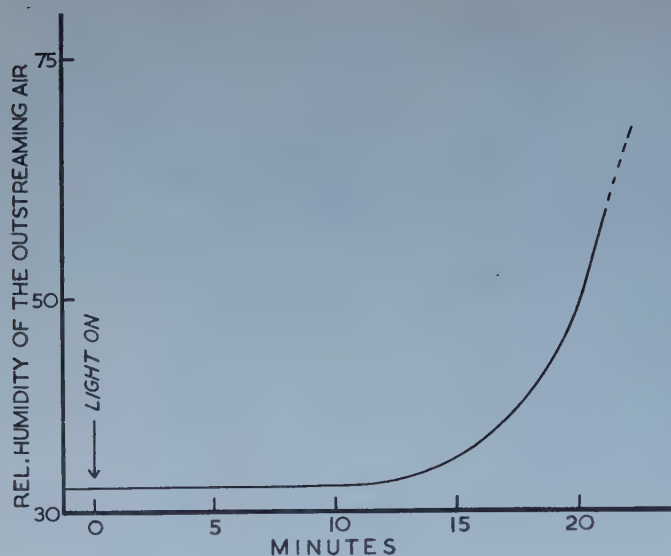
Figure 4. *The transpiration from wheat leaves illuminated with 64,000 meter candles.* Before illumination the leaves had been in darkness for 3 hours. (Cf. Figure 3).

humidity of 30 per cent. In light (64,000 meter candles) the maximum values for 10 plants with an average fresh weight of 1.1 g amounted to 70 per cent. The speed of the air stream was 800 ml/min., and the temperature in the chamber 22° C. Applying the general gas law for calculation of the amount of water transpired in light and darkness, the cuticular transpiration will be 0.0003 g/min. or 0.016 g/hr. and g fresh weight. The corresponding values for the stomatal transpiration will then be 0.0058 and 0.311.

In this connection it should be pointed out that there is a gradient for the humidity of the air in the transpiration chamber. At the bottom of the chamber the humidity of the air has a value of 30 per cent and at the upper end the value depends on the rate of transpiration. From experimental point of view this means that the stomata may not be equally opened over the whole illuminated area as the aperture depends to some extent on the humidity of the surrounding air (Heath and Milthorpe, 13; Paetz, 16; Thut, 29). To which degree the results in the present case are influenced by this factor has not been investigated.

Experimental

The Opening and Closing Phases of the Stomatal Movement

1. *Effect of a short light period given under constant darkness.* In Figure 1 was shown the general course of the transpiration during the opening phase under different light intensities. The course of the transpiration when light

Figure 5. *The transpiration from wheat leaves during short periods of light (64,000 meter candles).* Prior to illumination the leaves had been in darkness for 30 minutes. The Figure is a combination of 7 single experiments. Transpiration value in darkness 32 %. Humidity of the incoming air 30 %. ↓ = light on. ↑ = light off.
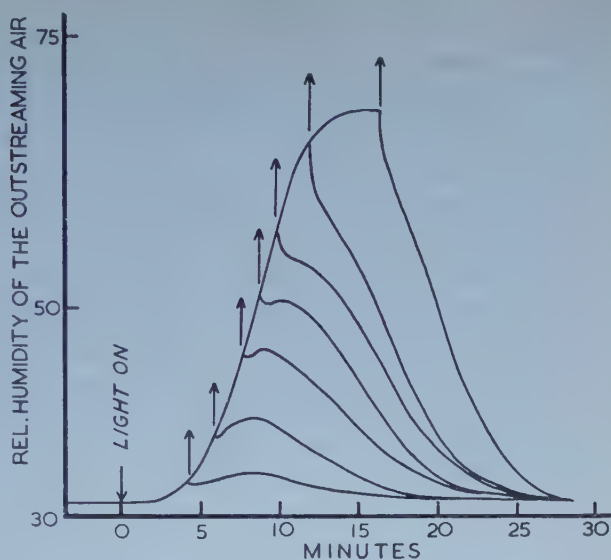
is given only for short periods of time can be seen in Figure 5. The Figure is a combination of 7 single experiments performed without changing the experimental material in the illumination chamber. Prior to the illumination the plants have been kept in darkness for 30 minutes in an air stream of 30 per cent relative humidity with a speed of 4 m/min. From the curves one can see that if the light is interrupted by darkness at the very beginning of the opening phase, the transpiration continues to increase and reaches its maximum 4 to 5 minutes later in darkness whereafter it decreases. This time delay becomes less and less evident with longer periods of illumination, i.e., when the stomatal aperture comes closer to its final value, but even if the light is interrupted at the constant part of the transpiration curve traces of the aforementioned time delay in the closing of the stomata are clearly demonstrable.

When the stomata are closed in darkness under optimal water deficit, they are under a pressure exerted by the surrounded epidermal and mesophyll cells. This pressure has to be overcome before the opening phase can begin. The time required for this to happen is presumed to be one of the courses for the expansion phase (Stålfelt, 23; p. 224 — experiments with Vicia Faba). As the photosynthetic mechanism begins to work immediately after the onset of illumination there is no reason to assume a delay in the formation of osmotically active substance, which is supposed to go via changes in pH (for review; see Small, 22). The increase in aperture of the stomata will begin when the diffusion pressure deficit of the guard cells has reacher and

begins to exceed the value for the mechanical pressure and suction forces exerted by the surrounding cells. According to Stålfelt (23) the counter-pressure is high in the beginning. During the middle phase of the opening movement it is lower and fairly constant. At the end of the phase the pressure increases again. At maximum aperture the newly formed osmotic substance must equal that which is disappearing per time unit. The way in which the counter-pressure can effect the enzymatic reaction is not known.

The equilibrium: non-osmotic substance $\rightleftarrows$ osmotic substance is at maximum aperture shifted so that the osmotic concentration has a high value (de Alvim, 1). Disregarding the way in which the osmotically active substance is formed in the guard cells one must count with one reaction which will always be a limiting factor in the attainment of equilibrium, namely, the diffusion of water into the stomata as a result of the change in diffusion pressure deficit. Even if the light may instantly cause a change in the content of osmotically active substances, which seems to be the case here, the attainment of equilibrium with the surrounding cells will be a slow process, determined mainly by the speed of the water diffusion. The delay in attainment of equilibrium will thus be greatest when the osmotically active substance formed per time unit is the greatest. This would explain the time delay in the stomata opening as represented in Figure 5.

At the moment when light is switched off one can observe a sudden decrease in the transpiration. This decrease is greater the greater the transpiration rate, i.e., the greater the aperture of the stomata. At the beginning of the opening phase the decrease is hardly noticeable but at a sudden onset of darkness when the transpiration rate has reached its maximum this decrease is great. A corresponding transient increase in the transpiration can be seen also when light is switched on after short periods of darkness following a previous light period. In the early stages of the experiments it was believed that these sudden changes were a physiological phenomenon connected with a rapid change in the light-dependent stomatal reaction. The fact, however, that the effect decreased with decreased aperture of the stomata pointed towards a purely physical phenomenon as the cause, namely, a sudden contraction or expansion of the intercellular air saturated with water vapor under the influence of darkness and light. The effect of these temperature changes will be greater the easier the air can pass from the leaves out to the surrounding air. If the light for illumination is not filtered, these changes are still greater; but even if the exciting light is filtered very carefully, so that it contains no or very small amounts of light of wave lengths over 7,000 Å, it was not possible to avoid this phenomenon completely.

All transpiration curves given in this paper are reproduced in their actual
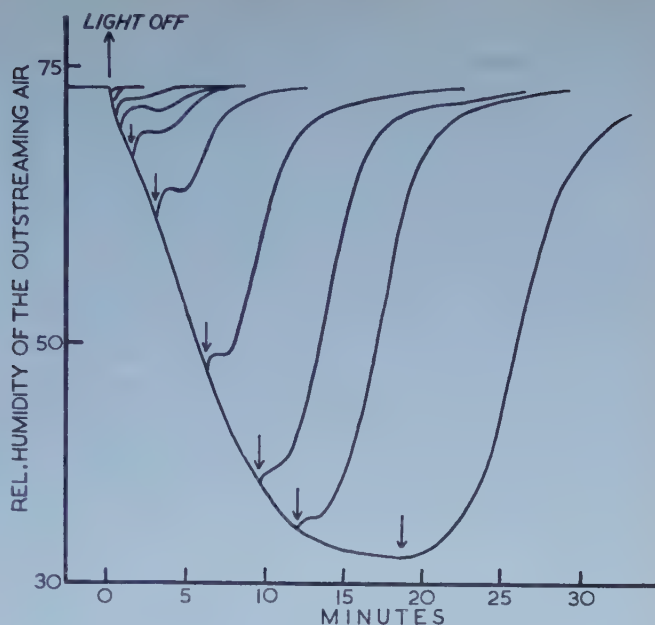
Figure 6. *The transpiration from wheat leaves during short dark periods. Prior to darkness the leaves had been in light for 30 minutes. The Figure is a combination of 10 single experiments. Otherwise as in Figure 5.*

shape without corrections for these aforementioned factors. This phenomenon. however. does not change anything in the discussion of the transpiration curves. It is probable. however, that the first physiological change in stomatal width as a result of the change from darkness to light or the reverse does not come as suddenly as indicated in the Figures.

2. *Effect of a short dark period given under constant illumination.* Closure of the stomata can be brought about by darkness (photoactive reaction) and by a decrease in the water content (hydroactive reaction). Of these two reactions the photoreaction seems to be more rapid. Though it is difficult to say how rapid the response is to an increase in the water deficit, the change in water content will for natural reasons always be considerably slower than the shift between light and darkness.

In Figure 6 is shown the change in transpiration when the constant illumination is interrupted by varying periods of darkness given when the transpiration has reached its maximum value. This takes at least 30 minutes in continuous light of an intensity of 64.000 meter candles. The Figure is a combination of 10 single experiments. The different parts of the descendent curve completely cover each other when the single experimental curves are brought together. The reproducibility of the experiments can be seen in Table 1, where the values for the humidity of the air are given for single experiments after different periods of time. These experiments are all per-

19

Table 1. *The values for the humidity of the outstreaming air following different periods of darkness after 30 minutes of light.* The units are arbitrary (the value for light=0; the value after 30 minutes in darkness=100).

Time after beginning of darkness	Humidity of the air						
	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI	Exp. VII
48 sec.	9.55	8.3	8.3	8.1	8.1	7.6	8.3
96 sec.	—	14.6	15.2	13.4	13.3	13.2	13.4
3 m. 12 s.	—	—	28.0	26.1	27.2	25.4	25.4
6 m. 24 s.	—	—	—	60.5	57.2	53.5	52.8
10 m.	—	—	—	—	81.5	79.5	79.5
12 m. 48 s.	—	—	—	—	—	91.0	89.0

formed with the same portion of plants and only light conditions and the age of the plants have been changed during the experimental time. During the course of the experiments the average length of the leaves increased 0.5 cm.

When light is switched óff one can notice a sudden decrease in the transpiration. It is not possible to decide the respective part of this decrease caused by the aforementioned physical phenomenon and by the pure physiological change. From what happens when light is switched on again the conclusion can be drawn that the change in relative humidity of the air as a result of the heat expansion amounts to about 2 per cent when the stomata are open and less than this when they are more or less closed.

As long as light is off the descendent part of the curve corresponds to that shown in Figure 5. Thus the sudden decrease in transpiration comes first. A few minutes later a certain slowing down of the decrease is noticeable conditioned by the time delay in the water diffusion into the guard cells — already discussed in connection with the experiments concerning the opening phase.

When light is switched on after different periods of darkness the appearance of the response obtained seems to be related to the length of the dark period, i.e., to the actual turgor of the guard cells and thereby to the stomatal aperture. If darkness has lasted for only a few minutes, the recovery proceeds rather slowly. If the dark period has lasted for about 5 minutes a prolongation of the same will not principally change the shape of the recovery curve. The course of the recovery is characterized by three distinctly different parts:

1. A rapid transient increase in the transpiration due to expansion of the intercellular air by heating.

2. A period of constant transpiration lasting for about 1—2 minutes due to new formation of osmotically active substance and a regaining of turgor by a water uptake, the speed of which is limited by the water diffusion into

the cells. This part of the curve can thus be directly compared with the extension phase (Stålfelt, 23) at the beginning of the light reaction It will also gradually pass over to this the lower down we come on the transpiration curve when also the phenomenon under point 1 will be less noticeable.

3. An increased transpiration which to all parts coincides with and corresponds to the general stomatal opening phase already discussed.

The complete agreement in slope between the different ascendent curves from the different experiments is very noticeable and points to the fact that the light — dark reaction is fully reversible. All these different experiments have been performed successively and as soon as the transpiration has reached the maximum value, a new period of darkness of different length has been applied. During these short periods of darkness and light adaption phenomena of any greater magnitude do not seem to occur (cf. Table 1.). The complete reversibility of the reaction is also evident in the experiments with intermittent light reported below.

Experiments with Intermittent Illumination

According to Stålfelt (23) the response of the stomata to light follows the product law, i.e., the attainment of a certain aperture is determined by the amount of light. The main light reaction would thus be of a photochemical nature. Harms (10) confirmed these results but stated that the product law is valid only within narrow limits of stomatal apertures and the good agreement in Stålfelt's experiment was due to a lucky chance in that just a certain aperture was chosen for the measurements. Due to the great reproducibility of experiments performed with the corona-hygrometer it would be possible by means of this apparatus to get more information concerning the validity of the product law. The interest was especially concentrated around the question whether the same result would be obtained if the light energy is administered intermittently or continuously. The experiments were arranged so that the response to continuous light was compared to the response to light of the double intensity given intermittently with dark periods of the same length. The reason for carrying out such experiments was to find out if the "yield" — in this case the transpiration — would increase if the formation of osmotically active substance in the guard cells — in light — is differentiated from the uptake of water — in darkness. Analogously to the finding that flashing light increases the yield in photosynthesis as compared to continuous light due to a separation of the light and dark reactions. (Emerson and Arnold, 6) one might expect some differences.

In Figure 7 is shown the slope of the opening phase — measured as transpiration — during an illumination with 64,000 meter candles (curve No. 1)
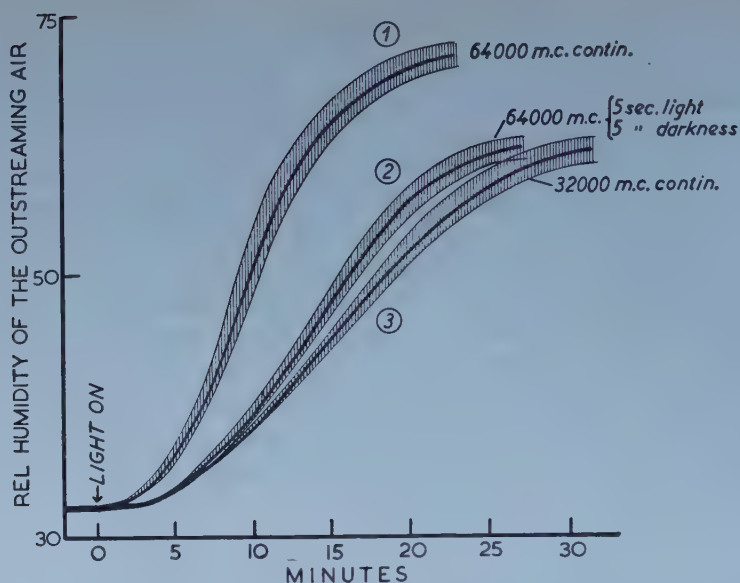
Figure 7. *The transpiration from wheat leaves in continuous and intermittent light.* Prior to illumination the leaves had been in darkness for 30 minutes. 1. Continuous illumination, 64,000 meter candles. 2. Intermittent illumination (5 sec. light, 5 sec. darkness), 64,000 meter candles. 3. Continuous illumination, 32,000 meter candles. Each series was repeated 6 times. The spread of the single curves are denoted with the thin curves.

as compared with the transpiration obtained at 32,000 meter candles (curve No. 3). Curve No. 2 shows the transpiration obtained if 64,000 meter candles are given intermittently in periods of 5 seconds interrupted by 5 seconds of darkness. From the curves it is evident that if the light is given intermittently, the transpiration is increased to some degree. This increase is always present. The maximum increase which can be obtained by giving light intermittently amounts to around 10 per cent of the value for continuous light. The increase in transpiration is restricted to the opening phase, whereas the end value is the same in the two cases. From the experiments mentioned here it is thus evident that intermittent light increases the speed at which the stomata open. This is also what should be expected from the experiments described above concerning the time lag between the end of illumination and establishment of osmotic equilibrium. In darkness the stomata will continue to open until the equilibrium is reached. During the new light period more osmotic material will be formed and so on. The increase depends on the length of the light and dark periods as can be seen in Figure 8. The Figure shows the opening phase under intermittent light with varying periods of intermittency as compared to continuous light with half of the former
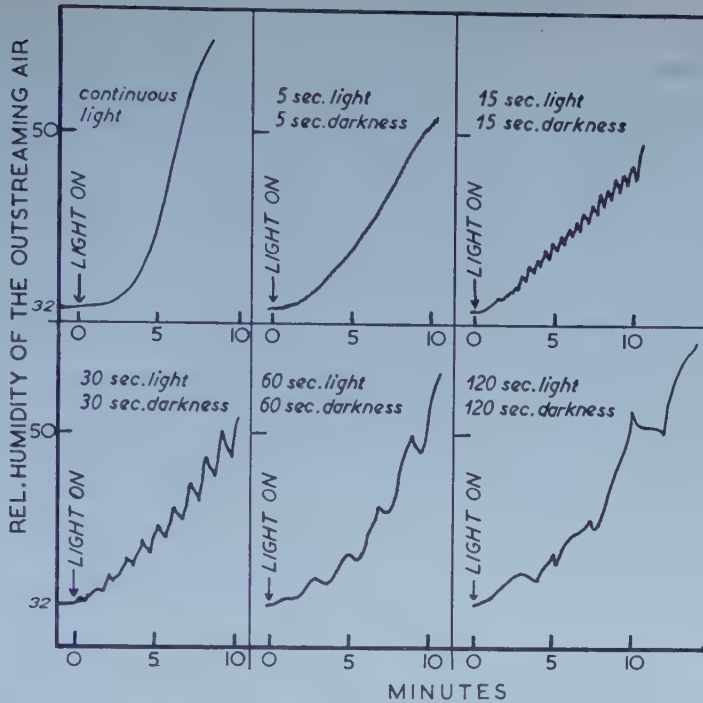
Figure 8. *The transpiration from wheat leaves during the first period of illumination after being in darkness for 30 minutes.* Continuous and intermittent illumination.

intensity. From the Figure it is evident that the greatest yield will be obtained with the shortest intervals, in this case 5 seconds. According to Figure 5 the maximum aperture is obtained 3—4 minutes after the light has been switched off. It might be possible, however, that the real maximum comes earlier, as the change resulting from the temperature effect might cause a small distortion of the true course of the process. It is therefore possible that an additional increase in the yield would be obtained by a further shortening of the periods. When the periods exceed 60 seconds, the irregularities of the curves become so great that it is difficult to get a definite value for the average slope.

As to the validity of the product law the experiments have given varying results. The experimental results presented in Figure 7 show that illumination with 32,000 meter candles of continuous light gives proportionally higher transpiration values as compared to the transpiration in the double intensity (curve No. 1). Other experiments, on the other hand, for instance those presented in Figure 1, show a fairly good agreement with the product law. A comparison of the angles between the curves and the time-axis in the

latter case shows that the angle for 64,000 meter candles is nearly twice that formed by the curve for 32,000 meter candles and the time-axis in this case. This is thus an indication that the product law is valid in this case. In this connection it is to be noted that the different series represented in the Figures are performed with the same material in the illumination chamber. The plant material is thus the same in different light intensities in the respective experiments. No closer studies have been made as to the frequency of the different kinds of response, but there are some evidences suggesting that the stage of development of the plants play a rôle in this respect. All the plants used in these experiments were of the same age, it is true. but due to a fault in the thermostatic mechanism in the cultivation chamber on one occasion the plants employed for the experiments shown in Figure 7 were in a later stage of development than those in Figure 1. A more detailed report of this, will be given in a following paper. The differences found in the transpiration from plants illuminated with intermittent light as compared to that in continuous light of half the intensity, however, are independent of the age of the plants.

During the course of these experiments with intermittent light the question arose as to whether there would be any stage of equilibrium for the transpiration, if the plant is illuminated with intermittent light for a longer period of time. A series of experiments were performed where the illumination was given intermittently — on the one hand starting from complete darkness (Figure 9 A), on the other from the time the maximum transpiration was reached in constant illumination (Figure 9 B). The experiments were performed during a period of 60 minutes, but in the Figures the changes during 45 minutes are reported — this period turned out to be sufficient for obtaining an equilibrium. The results from these experiments can be summarized as follows:

When light is given intermittently the transpiration fluctuates around a value which fairly well corresponds to the end value which should be obtained, were the plants illuminated continuously with a light of half the intensity employed in the intermittent illumination. The same value for the average transpiration will thus be obtained regardless of where the intermittent illumination has started.

Even the length of the periods of light and darkness does not seem to play any rôle for the establishment of the average value around which the transpiration fluctuates, provided the periods are of the same length. This is shown by the experiments presented in Figure 10, where the periods of light and darkness have been 1.5, 3, and 6 minutes respectively. In these experiments the intermittent illumination was started when the transpiration had reached
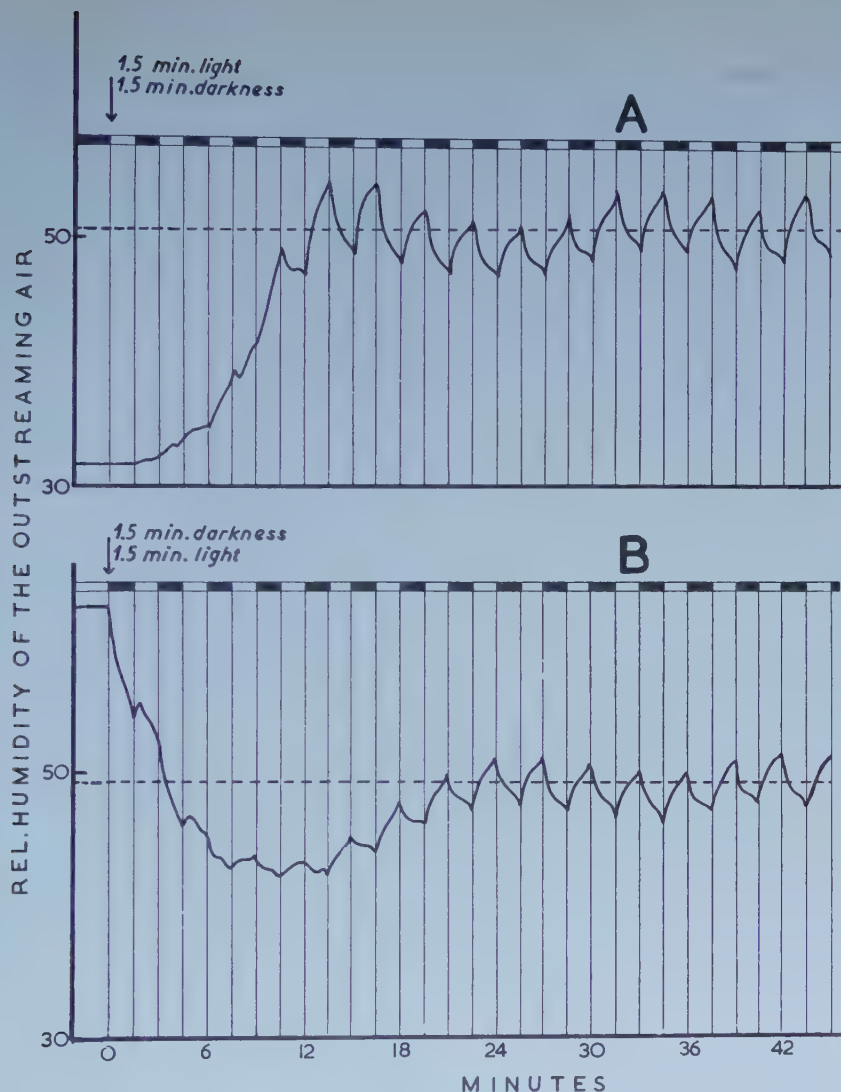
Figure 9. *The transpiration from wheat leaves in intermittent light.* A. After being in darkness for 30 minutes. B. After being in light for 30 minutes.

one and the same value in all experimental series. The experiments have been performed with the same plants without any change in the illumination chamber. One can see that before the equilibrium is reached the average transpiration fluctuates until this value is established. These fluctuations are characterized by the same general pattern as that obtained when light is given continuously. They are comparatively large at the beginning and become

gradually smaller and smaller (cf. Andersson *et al.* 3). It takes about 30 minutes from the start of the intermittency until equilibrium is established.

Some further conclusions can be drawn from these experiments. The fact that an equilibrium is reached at the value corresponding to the value for half of the light intensity given continuously shows that this equilibrium is established so that the speed of the increase in transpiration in light is the same as the speed of the decrease in darkness. If changes in transpiration originate from changes in stomatal aperture and these changes are related to changes in turgor in the guard cells, one may thus draw the conclusion that in intermittent light the rate of formation of osmotically active substance in light equals the rate of decomposition in darkness.

Effects of Intermittent Light Periods of Short Duration on the Development of Plants

Garner and Allard (7) studied the effect on the growth of plants of alternating equal periods of light and darkness varying in duration from 5 seconds to 12 hours. They noted a well-marked maximum in respect to the flowering in the neighbourhood of one minute alternations, vegetative development here showing a very definite minimum. Later on Gregory and Pearse (9) demonstrated that short alternating periods of light and darkness lead to partial closure of stomata in Pelargonium zonale, again showing a maximum aperture in the neighbourhood of one minute. Portsmouth (18) raised cucumbers under constant conditions of temperature and humidity with three different types of illumination: 12-hour alternations of light and darkness, 1-minute alternations and continuous light. The values for total leaf surface and stem length under very short periods (1-minute) of alternate light and darkness were found to differ markedly from the normal exponential curves obtained with long-period alternations or with continuous light. In the first case the relative growth rate of the leaf surface falls with time, whereas in the other two cases the relative rate remains constant. The curve of increase in stem length was sigmoid in the first case and exponential in the others. The main effect of variation in the period of alternations is found to be due to variation of net assimilation rate per unit area and can thus probably be referred to the aperture of the stomata. Similar experiments have recently been reported by Bonde (5) with young tomato and cocklebur

Figure 10. *The transpiration from wheat leaves in intermittent light.* After being in darkness for 30 minutes, continuous light was given until a certain transpiration value was reached, equal in all series. The intermittency was: A: 1.5 min.; B: 3 min.; C: 6 min. Light intensity 64,000 meter candles.
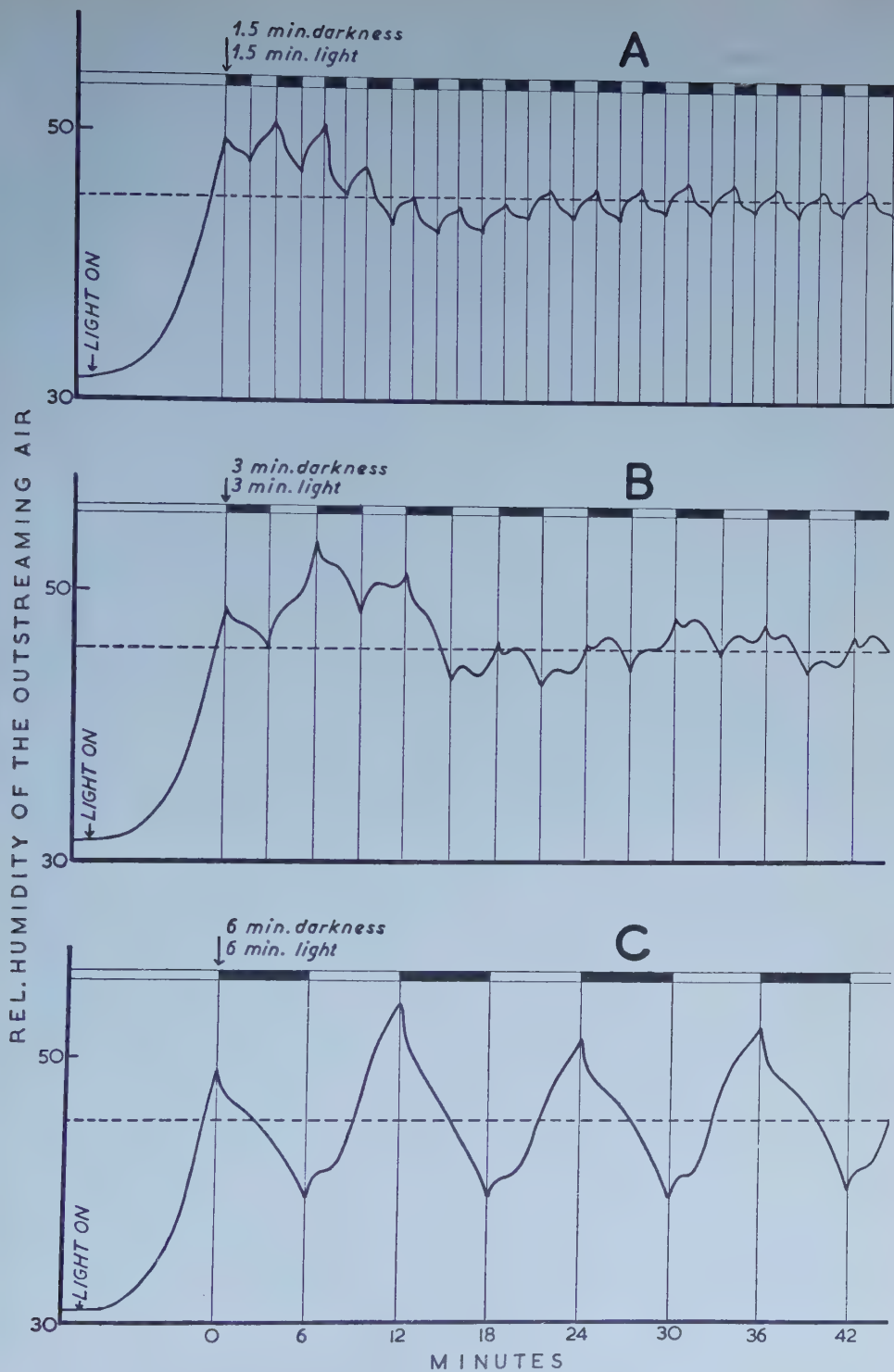
plants. Using cycles of different length (5, 15, 60 min. and longer) he found a greater production in respect to dry weight in 5 minute cycles as compared to that obtained in 15 minute. The greatest yield was found at the 1-hour cycle plants. For maximum formation of inflorescences still longer cycles had to be administered.

In respect to the behaviour of the stomata under conditions of alternating light and darkness there are only a few observations. Portsmouth (18) suggests that the difference in yield at different cycles of light and darkness is due to varying stomatal apertures. Gregory and Pearse (9) used the porometer method for a study of the stomatal aperture at alternating periods of light and darkness in order to find out whether there could be any relationship between the findings by Garner and Allard (7) and the behaviuor of the stomata in intermittent light. They found that with each duration of alternating light a fairly definite equilibrium position was reached, and this was the same whether approached from the fully closed or open position of the stomata. They also found that at equilibrium a minimum opening is obtained with alternations of 45 seconds' duration. With both longer and shorter alternations the stomata at equilibrium have a larger aperture.

A comparison of Gregory and Pearse's (9) experiments with those reported in this paper shows that the time required to open the stomata and to reach equilibrium was much longer in their experiments. This is probably due to the fact that they used rather low light intensities.

The experiments reported in this paper lend no support to any particular intermittency being specially favourable for obtaining any certain aperture of the stomata. They all seem to work so that a certain constant average value for the stomatal aperture is obtained, independent of the length of the periods of light and darkness.

The experimental object employed by Gregory and Pearse (9) consisted of leaves from Pelargonium zonale. The plant was cultivated under natural conditions in a greenhouse. The wheat plants used in the present investigation were cultivated under constant light and temperature. It is therefore possible that the autonomous rhythm found in the stomatal aperture reported by Gregory and Pearse in their Figure 3, p. 8 is the result of the pretreatment. The closing reaction in Pelargonium shows a considerable lag period which varies in extent, but is not less than one minute, while the opening reaction begins almost instantaneously. Presuming that the effects of these reactions are additive, "this would lead inevitably to the result that from position of maximum closure under alternating light the stomata would open, although at different rates with different periods of light and darkness" (Gregory and Pearse, 9, p. 9).

Differences in the rate of speed of the opening reaction as compared to the

closing reaction, resulting in an exceeding of the "average value" are indeed also clearly shown at the beginning of the periods of intermittency illustrated in Figures 9 and 10 in the present paper. The opening reaction in light proceeds faster here than the closing reaction in darkness when beginning from darkness and *vice versa*, when beginning from light. After some time, however, an equilibrium is attained, meaning that at this equilibrium the closure takes exactly the same time as the opening. This will then continue for at least 3 hours. The different behaviour of wheat as compared to the experimental material such as Pelargonium. tomato and cocklebur may, therefore, be due to lack of autonomous rhythms in wheat, which also is known to be a day-neutral plant.

Discussion and Conclusions

The experimental results reported in this paper indicate that the formation of osmotically active substances in the guard cells of wheat stomata under the influence of light is a rapid process. The rate of speed of the formation exceeds that of the diffusion of water into the guard cells due to the change in the diffusion pressure deficit. This means that the water diffusion to some extent is a limiting factor in the establishment of osmotic equilibrium.

The very rapid change in concentration in osmotically active substances leads to the consequence that the mechanical condition of the guard cells and surrounding cells must have a great influence on the speed of the movements and probably is another limiting factor for the speed of the change. The resistance exerted by the surrounding cells and also by the guard cells themselves against further enlargements of the aperture have been shown to be greatest at the beginning and and of the opening phase (Stålfelt, 23). This is probably the cause of the fact shown in Figure 1 that the time required to reach the maximum rate of acceleration in transpiration increases when lower light intensities are administered. The rate of formation of osmotically active substance is diminished by lowering the light intensity. The time necessary to overcome the initial resistance of the surrounding cells will then increase as the change in water pressure deficit proceeds more slowly in weak light as compared to higher light intensities.

The invalidity of the product law during the extension phase, especially shown by Harms (10), can thus be attributed to the fact that the mechanical resistance against changes in the shape of the guard cells is presumed to be greatest at these phases of the movement. Since these mechanical influences are due to many different factors such as water conditions of the cells, including colloidal conditions of the protoplasm (Weber, 30), it is reasonable

to assume that the influence of these factors will cause divergences from the product law. Only when the mechanical resistance against opening movement is the same during a certain period of time of the opening process, can the reaction be expected to obey the law; cf. Stålfelt, 23, p. 244. It is therefore understandable that experiments with different plants of different age can give varying results in this respect.

Due to the complexity of the reaction there are several adaption phenomena taking part in the process. Changes in the reaction time, i.e., variations in the length of the extension phase, are connected with pretreatment in respect to light as has been demonstrated by the de Alvim (1) and the author in this paper. These variations have been shown to be connected to variations in the content of photosynthetic material in the guard cells as starving increases the time (de Alvim, 1). Also the pressure conditions between the separate cells in the epidermic layers effect the extension phase, as the resistance exerted on the guard cells has to be overcome before the opening can start (Stålfelt, 23). Other adaption phenomena — more prolonged than the light induced ones — are related to the water conditions in the whole plant. Supraoptimal deficit has thus an after-effect consisting of a decrease for some time in the sensitivity of the guard cells to light (Stålfelt, 28). These two main adaptation phenomena do not seem to play a great rôle, however, in short illumination experiments, provided the experiments are performed within a short period of time and the water content of the plant is kept at an optimal level. This has clearly been shown in this paper and makes possible the use of the method described for quantitative measurements of the influence of light on the stomatal apparatus.

In the experiments with intermittent light it was found that after a certain period of illumination the time for building up osmotic material in light exactly equals the time required for the decomposition of the same amount in darkness. This finding shows that with any intermittency of equal periods of light and darkness, an equilibrium will be attained and that no after-effects can be registered. This means that the reaction is completely reversible, at least within short periods of time.

Summary

The behaviour of the stomata of wheat leaves in light and darkness has been studied indirectly by measuring the transpiration. For the determination of transpiration the new method with the corona-hygrometer described by Andersson et al. (3) and Andersson and Hertz (2) has been used.

The main results are:

1. The transpiration continues to increase for a few minutes in darkness given in the middle of the opening phase in light. This indicates that the formation in light of osmotically active substances is a rapid process as the time lag is presumably due to the diffusion of water into the guard cells. This will become the limiting factor at the establishment of osmotic equilibrium.

2. The closing phase of the stomata movement sets in within 15 seconds after darkness. If light is given again a considerably longer period of time is required to retain the original aperture.

3. Intermittent light produces a more rapid increase in transpiration, *i.e.*, more rapid stomatal opening, than the same amount of light given continuously. The explanation for this is the aforementioned time delay caused by the water diffusion.

4. In plants illuminated with intermittent light for longer period of time an equilibrium is established characterized by a formation of osmotically active substance in light which equals the amount of substance decomposed in darkness. This takes place provided the light and the dark periods are of the same length. In this connection it is insignificant whether the intermittency begins after the plant has been kept in continuous light with open stomata or has been in darkness with closed stomata.

References

1. de Alvim, P. T.: Studies on the mechanism of stomatal behaviour. — Amer. J. Bot. 36: 781. 1949.
2. Andersson, N. E. & Hertz, C. H.: Positive Spitzenentladung als Hygrometer geringer Trägheit. — Zeitschr. f. angew. Physik 7: 361. 1955.
3. — — & Rufelt, H.: A new fast recording hygrometer for plant transpiration measurements. — Physiol. Plant. 7: 753. 1954.
4. Bange, G. G. J.: On the quantitative explanation of stomatal transpiration. — Acta Bot. Neerl. 2: 255. 1953.
5. Bonde, Erik K.: The effect of various cycles of light and darkness on the growth of tomato and cocklebur plants. — Physiol. Plant. 8: 913. 1955.
6. Emerson, R. & Arnold, W.: A separation of the reactions in photosynthesis by means of intermittent light. — J. Gen. Physiol. 15: 391. 1932.
7. Garner, W. W. & Allard, H. A.: Effect of abnormally long and short alternations of light and darkness on growth and development of plants. — J. Agr. Res. 42: 629. 1931.
8. Gregory, F. G., Milthorpe, F. L., Pearse, H. L. & Spencer, H. J.: Experimental studies of the factors controlling transpiration. II. The relation between transpiration rate and leaf water content. — J. Exp. Bot. 1: 15. 1950.

9. Gregory, F. G. & Pearse, H. L.: The effect on the behaviour of stomata of alternating periods of light and darkness of short duration. — Ann. of Bot. N.S. 1: 3. 1937.

10. Harms, H.: Beziehungen zwischen Stomataweite, Lichtstärke und Lichtfarbe. — Planta 25: 155. 1936.

11. Heath, O. V. S.: Studies in stomatal behaviour. II. The role of starch in stomata. Part I. Review of literature, and experiments on the relation between aperture and starch content in the stomata of Pelargonium zonale. — New Phytologist 48: 186. 1949.

12. — Studies in stomatal behaviour. II. The role of starch in the light response of stomata. Part II. The light response of stomata of Allium Cepa L., together with some preliminary observations on the temperature response. — New Phytologist 51: 30. 1952.

13. — & Milthorpe, F. L.: Studies in stomatal behaviour. V. The role of carbon dioxide in the light response of stomata. Part II. Preliminary experiments on the inter-relations of light intensity, carbondioxide concentration, and rate of air flow in controlling the movement of wheat stomata. — J. Exp. Bot. 1: 227. 1950.

14. Hygen, G.: Studies in plant transpiration. I. — Physiol. Plant. 4: 57. 1951.

15. Liebig, Margarete: Untersuchungen über die Abhängigkeit der Spaltweite der Stomata von Intensität und Qualität der Strahlung. — Planta 33: 206. 1942.

16. Paetz, K. W.: Untersuchungen über die Zusammenhänge zwischen stomatärer Öffnungsweite und bekannten Intensitäten bestimmter Spektralbezirke. — Planta 10: 611. 1930.

17. Pisek, A. & Winkler, E.: Die Schliessbewegung der Stomata bei ökologisch verschiedenen Pflanzentypen in Abhängigkeit vom Wassersättungszustand der Blätter und vom Licht. — Planta 42: 253. 1953.

18. Portsmouth, G. B.: The effect of alternate periods of light and darkness of short duration on the growth of the Cucumber. — Ann. of Bot. N.S. 1: 175. 1937.

19. Rufelt, H.: Influence of the root pressure on the transpiration of wheat plants. — Physiol. Plant. 9: 154. 1956.

20. Scarth, G. W. & Shaw, M.: Stomatal movement and photosynthesis in Pelargonium. I. Effects of light and carbon dioxide. — Plant Physiol. 26: 207. 1951.

21. Sierp, H.: Untersuchungen über die Öffnungsbewegungen der Stomata in verschiedenen Spektralbereichen. — Flora 128: 269. 1933.

22. Small, J.: pH and Plants. — New York 1946.

23. Stålfelt, M. G.: Die photischen Reaktionen im Spaltöffnungsmechanismus. — Flora N.F. 21: 235. 1927.

24. — Die Abhängigkeit der Spaltöffnungsreaktionen von der Wasserbilanz. — Planta 8: 287. 1929.

25. — Der Einfluss des Windes auf die kutikuläre und stomatäre Transpiration. — Sv. Bot. Tidskr. 26: 45. 1932.

26. — Der stomatäre Regulator in der pflanzlichen Transpiration. — Planta 17: 22. 1932.

27. — Die Transpiration und Kohlensäureassimilation bei Blättern und Stroh des Hafers. — Angew. Bot. 17: 157. 1935.

28. — The stomata as hydrophotic regulator of the water deficit of the plant. — Physiol. Plant. 8: 572. 1955.

29. Thut, H. F.: The relative humidity gradient of stomatal transpiration. — Amer. J. Bot. 26: 315. 1939.

30. Weber, F.: Cytoplasma- und Kernzustandänderungen bei Schliesszellen. — Protoplasma 2: 305. 1927.

31. Williams, W. T.: Studies in stomatal behaviour. I. Stomatal movement induced by heat-shock and transmission of such stimuli across the leaves of Pelargonium zonale. — Ann. of Bot. N.F. 12: 35. 1948.

32. — Studies in stomatal behaviour. III. The sensitivity of stomata to mechanical shock. — Ann. of Bot. N.S. 13: 309. 1949.

33. — Studies in stomatal behaviour. IV. The water-relations of the epidermis. — J. Exp. Bot. 1: 114. 1950.

34. — & Barrett, F. A.: The effect of external factors on stomatal starch. — Physiol. Plant. 7: 298. 1954.

35. Yemm, E. W. & Willis, A. J.: Stomatal movements and changes of carbohydrate of Chrysanthemum maximum. — New Phytologist 53: 373. 1954.

The Xylenol Method and Determination of Nitrate in Beets

By

CHRESTEN SØRENSEN

Department of Soil Fertility and Plant Nutrition,
Royal Veterinary and Agricultural College, København
(Received March 12, 1956)

I. Introduction

The xylenol method for determination of nitrate in plants and soil was devised in 1929 by Blom and Treschow (6). The method is based on nitration of 2 : 4 xylene-1-ol to 6-nitro-2 : 4-xylene-1-ol (nitroxylenol).

In 1933 the method was examined by Alten and Weiland (1) who showed that nitroxylenol in solution obeys Beer's law. In the same year Treschow and Gabrielsen (15) revised the method which has subsequently been modified by several other authors and used for such varied purposes as analyses of air contaminated by nitrate groupings (16), analysis of vegetable matter (2), and for the determination of nitrate in salted meat (3). A description of the method is also given by Snell and Snell (14) who depend chiefly on Hamy's investigations (10).

In the following a brief account will be given of the procedure described by Treschow and Gabrielsen:

To a sample of 0.1—1 g of fresh vegetable material or of 25—50 mg of dried material (moistened with a few drops of water) add 25 ml of 66 % v/v sulphuric acid and 0.1 ml of xylenol. Stopper the reaction flask and after vigorous shaking set it aside at room temperature for 15—30 minutes for nitration; then add 100 ml of tap water and shake. Within a period of 48 hours distill off the nitroxylenol formed. As receiver use a 100 ml measuring flask containing 25 ml of 0.2 N NaOH; distill off about 70 ml, fill up to the mark with water, cool to 20° C, and make the colorimetric measurement within 30 minutes.

The addition of NaOH to the nitroxylenol presumably causes an electronic rearrangement in the molecule to form a quinonoid-type ion, as in the case of o- and p-nitrophenol. This ion is of an intense orange-yellow colour.

Since the method devised by Treschow and Gabrielsen (15) appears to be fairly convenient and rapid, it was attempted to use this method for the determination of nitrate in fodder sugar beet. However, it was quickly realized that the method was not quite satisfactory for this purpose, as the nitrate percentage found varied with the quantity of material used for the analysis, and, furthermore, it was inconvenient to have to make the colorimetric measurement within half an hour. As the method, however, offered the advantage that it only required small quantities of material, it was made the object of a more detailed investigation in order to render it suitable for routine analyses of root crops.

II. General Investigations

Apparatus and chemicals. Nitration was performed in 250 ml flat-bottomed pyrex flasks. The arrangement of the distillation apparatus was as described by Treschow and Gabrielsen (15). A Coleman Universal Spectrophotometer, model 14, was used for the colorimetric determinations, which — where nothing else is stated — were made at λ 460 mμ.

The chemicals were all of the quality denoted "pure" or "analytical grade". The applied dilutions of sulphuric acid are denoted by the content of ml of conc. sulphuric acid per 100 ml solution; the same applies with regard to the ethanol solutions.

The sugar beet samples used for the experiments consisted of pulp which had been dried for 24 hours at 80° C and subsequently ground to a fine powder in a mortar.

Interference from various compounds. Blom and Treschow (6) showed that the presence of oxalic acid delays nitration, that the presence of H_2O_2 may result in too low values, and that glycine and asparagine after heating with potassium permanganate in conc. sulphuric acid produce a yield of nitroxylenol. Alten and Weiland (1) found that the presence of manganous sulphate and lead acetate reduced the yield of nitroxylenol somewhat while HCl and HBr reduced it considerably.

Barnes (5) found that NaCl in quantities exceeding 0.3 mg interfered in determinations of 5—10 μg of nitrate nitrogen.

Hamy (10) mentions that tannins interfered while the presence of reducing sugars has no influence; this is further emphasized by Rauterberg and Benischke (13) who also show that starch does not interfere. Yagoda (16) mentions that nitration should be discontinued after a period of 10 minutes if glycol is present. There is no doubt that proteins may interfere, a view which is substantiated by results of Alten et al. (2).

Holler and Huch (11) found that nitrite together with 3 : 4-xylenol forms coloured compounds which are able to distil with steam.

The investigations dealt with in the present work showed that to a nitration sample
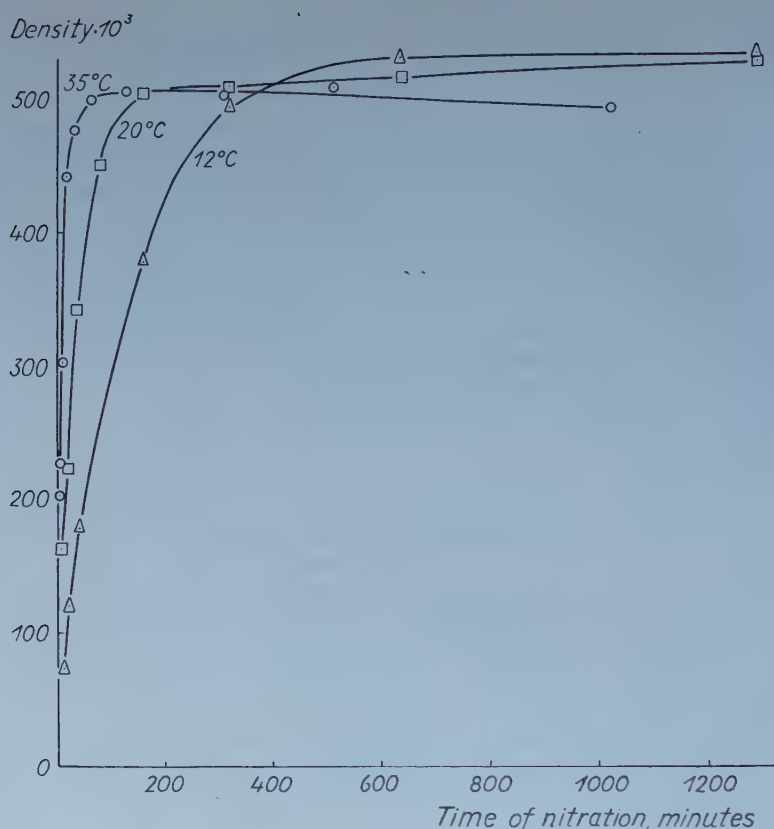
Figure 1. *Rate of reaction at different temperatures.*

containing 7 mg of NaCl it was necessary to add 100 mg of silver sulphate, but only 40 mg of silver acetate (in solution) to prevent a decrease in the nitroxylenol yield; presence of silverchloride precipitate did not interfere. Up to 1 ml of saturated copper sulphate solution in the nitration sample had no influence on the results.

The addition of 9 mg of tyrosine, 10 mg asparagine, 50 mg of betaine or 20 mg ammonium sulphate to the nitration sample resulted in an alteration of the nitroxylenol yield corresponding to about 1 µg nitrate-N.

When it was a question of small quantities of nitrate (10 µg nitrate-N) the presence of sucrose apparently resulted in a too high nitroxylenol yield, while the yield obtained became too low when it was endeavoured to determine e.g. 100 µg nitrate-N and 70 ml was distilled over as done in the original procedure.

Nitration. Time and temperature. The duration of the nitration suggested by different authors varies considerably, from 4 hours (1) to 20 minutes (2). Nitration temperatures of 20° C (10) and 50° C (1) have been applied, while one author states that the temperature must not exceed 35° C.

In order to get information about the rate of nitration at different temperatures

the following experiments were carried out. Flasks, containing 25 ml 66 % sulphuric acid and 0.1 ml xylenol, were immersed in a thermo-regulated water bath ($\pm 1°$ C), and left here for 15 minutes before nitration was started by the addition of 5 ml of potassium nitrate solution (0.5 mg N).

The results for some of the temperatures appear from figure 1. The rate of nitration increases with the temperature. At 12° C the nitroxylenol yield is constant for elongated time of nitration, while at 35° C the decomposition of nitroxylenol seems to be measurable in the course of few hours. However, other experiments on the rate of reaction showed that the nitration takes place more slowly when the substance to be analysed is of vegetable origin.

A heat effect of 14° C is obtained when 5 ml of an aqueous potassium nitrate solution is mixed with 25 ml 66 % sulphuric acid at about 20° C and the nitration flask is placed on the bench. Due to this heat effect the method is fairly independent of temperature in the surroundings.

Later experiments showed that if 3 ml of nitrate solution is used together with 22 ml of 75 % sulphuric acid per analysis, the nitration period of 40—60 minutes should be preferred at a room temperature between 12 and 28° C. The content of sulphuric acid in the nitration sample will then be about 66 %.

Concentration of sulphuric acid. The influence of the concentration of the sulphuric acid on the nitroxylenol yield was examined by Blom and Treschow (6) who, when the nitration process was allowed to proceed for 1 to 2 hours at 30° C, found the yield of nitroxylenol to remain the same within a concentration range from 55 to 70 per cent. The problem has subsequently been dealt with by Alten and Weiland (1).

The results of the present investigation agree very well with those obtained by Blom and Treschow (6) in the case of determinations of nitrate-N in potassium nitrate. When nitrate-N was determined in dried sugar beet pulp the relative nitroxylenol yield apparently increased considerably with increasing sulphuric acid concentration and decreasing weight of the sample analysed (120 to 30 mg). The reason for this seems to be that certain oxydative compounds pass into the distillate and disturb the photometrical measurement.

As it was found that the distillation of these side-products could be prevented, the use of a sulphuric acid concentration of 66 % was continued.

The significance of the vapour pressures of xylenol and nitroxylenol. Since xylenol is insoluble in sulphuric acid and able to distil in steam, the heat effect produced when tap water is added to the analysis may aid the evaporation of nitroxylenol. To illustrate the care with which the analysis should be made, a number of experiments was made using 500 µg nitrate-N per analysis. The following results were obtained. No measurable decomposition of xylenol takes place on standing in 66 % sulphuric acid for one hour. If a mixture of xylenol and sulphuric acid (in quantities corresponding to those used per analysis) is left for one hour in an unstoppered flask or when the nitration was allowed to proceed in an unstoppered flask for one hour or when the nitration mixture was left for one hour in an unstoppered flask after the addition of tap water, a reduction in the nitroxylenol yield amounting up to about 6 % was found. A mixture of 25 ml of 66 % sulphuric acid and 0.1 ml xylenol was set aside for 11 days, 500 µg nitrate-N was added and nitration took place; the yield of nitroxylenol obtained was about 18 % lower than the yield obtained when the three chemicals were mixed simultaneously.

Oxidation of xylenol in alkaline solution. Originally Blom and Treschow (6) stated

Table 1. *Influence of NaOH on the distillate from nitration mixtures with and without nitrate.* Optical density $\times 10^3$, measured at various times after distillation, the distillates being treated in different ways.

Distillate No.	μg No₃–N taken	NaOH added after distill. minutes	Spectrophotometric measurement minutes after distillation				
			10	40	70	130	600
1	0	128				4	21
2	0	128				5	21
3	10	128				61	76
4	10	128				62	73
5	0	8	4	6	8	10	23
6	0	8	3	6	7	11	23
7	10	8	60	62	64	66	77
8	10	8	61	62	64	67	78
9	50	8	257	259	260	262	270

that the spectrophotometric measurement could be made 30 minutes after the distillation. Later on it was shown by Alten et al. (2) that the optical density increases during the first 30 minutes after the distillation and then remains constant for a period of up to 5 hours. Rauterberg and Benischke (13) state that the alkaline distillate will keep for several days without showing any increase in the optical density.

As will appear from Table 1, the present investigations showed that a considerable increase in the optical density took place following the addition to the distillates of sodium hydroxide.

The increase in the optical density in alkaline solution is presumably caused by oxidation of xylenol. Both the temperature and the access of air seem to have a certain influence on this process. 0.1 ml xylenol + 25 ml 0.2 N sodium hydroxide diluted with water to a volume of 100 ml and heated to 50° C in the presence of air exhibited an optical density corresponding to 82 μg nitrate-N. Another mixture which was kept for 7 days at 20° C in the presence of air proved to have an optical density corresponding to 180 μg N.

Distillates in stoppered flasks containing nitroxylenol corresponding to 400 μg nitrate-N exhibited, in the course of 24 hours, an increase in optical density corresponding to 3 μg nitrate-N⁻ only and, after a further 10 days, an increase corresponding to 10 μg nitrate-N. In the case of distillates originating from dried sugar beet pulp containing about 300 μg N the increase was slightly greater. It should in this connection be borne in mind that the surplus of xylenol (which passes into the distillate) is less in the case of great quantities of nitrate than in the case of small nitrate quantities, this presumably explains the smaller increase in the density of the last mentioned solutions.

If distillates are stored in acid ethanolic solution no oxidation occurs. The optical density of distillates corresponding to 400 μg N and stored in a 10 per cent ethanol solution (pH = 3 to 4) remained unchanged for 48 hours, then a reduction commenced which after 19 days corresponded to 60 μg nitrate-N. The results seem to show that the increase in the optical density of the distillate on standing in alkaline solution is not caused by nitroxylenol which is in agreement with the result of Treschow and Gabrielsen (15).

Blom and Treschow (6) found that the intensity of the colour of a nitroxylenol solution increased when the temperature increased. In the present work it has not been possible to find any temperature dependence.

Quantity of xylenol. On account of the oxidation of xylenol in the alkaline distillate a reduction of the quantity of xylenol used would be desirable.

When using a xylenol mixture consisting of 1.016 g xylenol and 5 ml glacial acetic acid (nitration period: 3 hours, 500 µg potassium nitrate-N) the nitroxylenol yield remained unchanged when the amount of xylenol mixture was varied from 0.1 ml to 1.0 ml. When the analytical samples consisting of dried sugar beet pulp, the nitroxylenol yield increased by about 10 % when the amount of xylenol mixture was increased from 0.1 ml to 0.2 ml, but did not increase with a further increase of the mixture.

Addition of ethanol before the distillation and extraction with ether. Since xylenol is oxidized in a solution of sodium hydroxide and since the intensity of the colour of nitroxylenol is not sufficient to enable measurements to be made unless in alkaline medium, the problem of how to obtain minimum oxidation would have to be solved.

It might be imagined that addition of ethanol to the distillation sample would act as solvent for nitroxylenol in the distillate, and the tendency of nitroxylenol to crystallize on the walls of the condenser might be eliminated, the addition of sodium hydroxide to the distillate could thus be postponed until just before the colorimetric measurement. Examinations showed that the optical density of a nitroxylenol solution increased about $1 \frac{1}{2}$ per cent after addition of 5 ml ethanol, but if 5 ml ethanol was added to a mixture before distillation, the density was 11 per cent higher than it was when no ethanol had been added. This suggests either that a higher yield of nitroxylenol has been obtained or that together with ethanol other nitroxylenol derivatives than 6-nitro-2 : 4-xylen-1-ol will distill over. By an addition of ethanol reproducible results might be more readily obtained.

For the purpose of cleaning the condenser the method used in the present work appears to be convenient and by distillation into an empty receiver and subsequent flushing with 15 ml 20 % ethanol it is possible to eliminate measurable oxidation of xylenol. Addition of more than 5 ml of ethanol does not seem to present any advantages.

Another way in which it might be possible to eliminate oxidation of xylenol would be to extract nitroxylenol from the nitration mixture as preferred by Hamy (10) and Balks and Reekers (4) who used ether, while Barnes (5) used toluene for the extraction and found that sucrose interferes in the process of extraction. Extraction with ethyl ether was attempted with subextraction from the ether phase by means of sodium hydroxide. For the determination of nitrate in dried sugar beet pulp the method did not produce satisfactory results, the amount of nitrate found being dependent on the amount of dry matter used.

Blank determinations. The extinction curve of nitroxylenol has a maximum at about λ 450 mµ, but a blank determination made on dried sugar beet exhibits considerable absorption at this wavelength.

Figure 2 shows a set of absorption curves for distillates obtained from blank determinations. Some of the curves are drawn to a reduced scale to make a convenient comparison of their forms. The blank determinations have been made by carrying out the whole analysis as usual, the blank run on sucrose has been made both with and without the addition of xylenol. The curves represent mean values of two duplicate determinations.
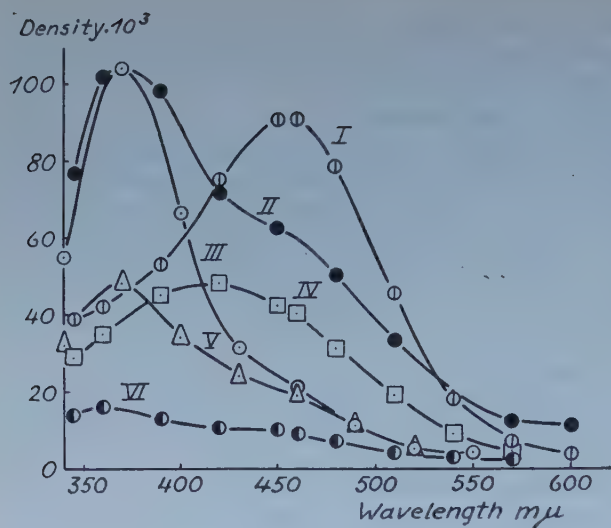
Figure 2. *Absorption curves of distillates from various blank determinations and of nitroxylenol.* Curve I arises from nitroxylenol. Curve II represents dried sugar beet and, Curve III sucrose to which xylenol has been added. Curve IV represents a blank determination made on xylenol and, Curve V sucrose to which no addition of xylenol has been made. Curve VI represents extract from dried sugar beet, the extract made by means of sodium hydroxide with copper sulphate added before filtration.

It can be seen from curves II, V, and VI that the absorption curves obtained from blanks run on dried sugar beet pulp, sucrose and extract of dried sugar beet pulp are very nearly similar while the absorption curve of sucrose to which xylenol has been added (curve III) shows a slight deviation. It is possible that xylenol reacts with sucrose or with compounds resulting from sucrose.

The quantitative value obtained from the blank determination was greatly dependent on the preparation of the analytical material. Distillates from blank determinations on 1 g of fresh beet pulp exhibiting a density corresponding to 8 µg nitrate-N while blank determinations made on 30 mg and 120 mg of dried sugar beet pulp corresponded to 3 and 4 µg nitrate-N. When distillation mixture in stead of 1 hour, was allowed to stand for three days, the result of the blank determination corresponded to 8 µg nitrate-N. If on the other hand an extract was made of dried sugar beet pulp (about 2 g) or a corresponding amount of beet juice with 44 ml of 0.2 N sodium hydroxide, 6 ml of saturated copper sulphate solution being added before filtration of the extract, the result of the blank determination on 5 ml filtrate was found to correspond to 1 µg nitrate-N. When, however, silver acetate, sodium hydroxide and copper sulphate were used for the extraction, as described in the following description of the analytical procedure, the optical density of blanks was found to be less than the experimental error of the determination.

Fractional distillation. When the process of distillation is observed it can be seen quite clearly that the major part of the nitroxylenol present distills over with the first few milliliters.

If the optical character of the last few ml of the distillate differs from that of nitroxylenol, and if coloured compounds are formed in the course of the distillation and pass over with the distillate, it is to be recommended to reduce the volume of distillate and the distillation time. To elucidate this a number of experiments were made with fractional distillation of blanks run on extracts of dried pulp. Quantities of 25 ml + 25 ml were distilled off, 50 ml of tap water was added, and 25 ml + 25 ml were distilled off — this was repeated so as to obtain altogether 6 fractions. The

Figure 3. *Absorption curves*. Fractions of distillates from:

A: potassium nitrate.

B: dried sugar beet pulp without treatment.
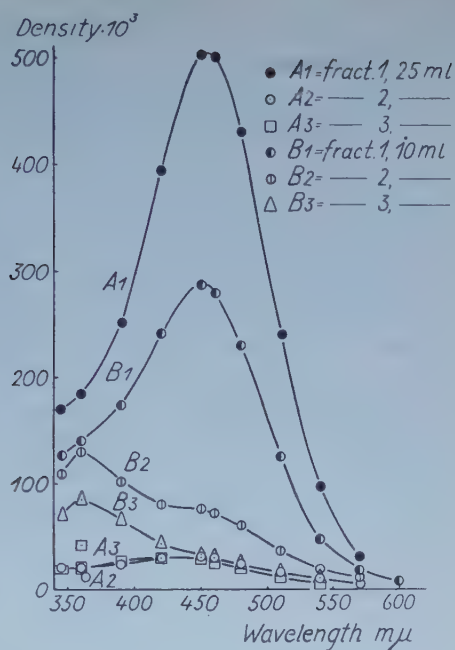
optical density (at λ 460 mμ) of the alkaline distillates was doubled from fraction I (0.011) to fraction II (0.021), while in the remaining fractions the density was slightly higher than that of fraction I, but fairly constant.

When the fractions were not made alkaline, the optical density was 0 or 0.001. The experimental results further showed that the density of the blank is influenced by the concentration of the sulphuric acid in the distillation flask, and that during the distillation a fairly continuous formation of volatile compounds seems to take place.

Experiments on the addition of different quantities of tap water did not indicate that any advantage was obtained by adding more than 75 ml of tap water to the nitration mixture.

Series of experiments were made in order to determine the optical nature of different fractions of nitroxylenol distillates from nitrate determinations on dried sugar beet pulp as well as on potassium nitrate. A few typical results from the experiments made are illustrated in figure 3. Nitration took place in a 66 % sulphuric acid medium.

To the mixture in the nitration flask A (the analytical material used being pure potassium nitrate) was added 100 ml tap water and three fractions of 25 ml each were distilled off and denoted A_1, A_2, and A_3. To the mixture in the nitration flask B (the analytical material used being dried sugar beet pulp) was added 75 ml tap water and fractions of 10 ml, 20 ml and 20 ml were distilled off and denoted B_1, B_2, and B_3. When the distillation was stopped, the sulphuric acid concentration was thus the same in both flasks. For the sake of clarity (figure 3) the observed densities have in the case of fractions A_2, B_2 and A_3, B_3 been multiplied by 3.

It appeared from all the experiments that the absorption curves of fractions "1"

Table 2. *Distribution of nitroxylenol between different fractions.*

Analytical material	µg N per analysis	Number of detm.	Tap water ml	Volume of fractions in ml			% total nitroxyl. in		
				a	b	c	fract. a	fract. b	fract. c
KNO₃	500	4	100	25	25	25	97.6	1.3	1.1
KNO₃	500	1	75	10	20	20	96.0	2.8	1.2
pulp treated with NaOH & CuSO₄...	50 & 100	2	75	10	20	20	98.4	0.9	0.7
pulp (without any treatment)	30—100	3	75	10	20	20	94.1	4.8	1.1

agreed with the absorption curve of nitroxylenol. Fractions "2", the absorption curves of which agreed closely with those of the blanks (see figure 2), represented a relatively larger part of the total optical density (the sum of 1, 2, and 3), when organic matter was present than when only pure potassium nitrate was present. This part of the total value increased when the amount of dry matter used for the analysis was reduced, while it decreased considerably when the analytical sample was treated with sodium hydroxide and copper sulphate. In the case of fractions "3" these phenomena were even more pronounced. Variations of the time allowed for the nitration and the time which elapsed between the addition of tap water and the distillation did not result in any alterations in the form of the absorption curves.

The present examinations show that when the analytical material is dried sugar beet pulp which has not been subjected to extraction, a measurable part of the density of the distillate is caused by the presence of substances other than nitroxylenol. As a way out the volume of distillate might be reduced or a correction for the blank value introduced as suggested by Alten et al. (2). The latter method seems not to be a satisfactory solution in the case of dried beet for which the blank value is considerable.

Table 2 summarizes some of the results which show the distribution of nitroxylenol between the different fractions of the distillate. KNO₃ and dried sugar beet pulp are used as analytical material. The results show that the rate of distillation seems to be smaller when dried beet material is used directly than when the said extract is used. The reason for this may be that nitroxylenol is adsorbed to the considerable quantities of colloids present when vegetable material is used directly. When 20 ml has distilled over, the rate of distillation of nitroxylenol seems to become small. This appeared also from the subsequent experiments since no difference in nitroxylenol yields could be demonstrated whether the volume of the distillate was 20 ml or 25 ml.

III. **Extraction Experiments and Determinations of Nitrate in Dried Sugar Beet Pulp**

As a certain disagreement was found to occur between duplicate determinations, and the nitrate percentages found were higher the smaller the

amount of pulp used for the analyses (dry matter moistened with water was used directly for the nitration), and as the blank value obtained from dry matter seemed to be unreasonably great, it was examined whether extraction of nitrate could be made in such a manner that these drawbacks were eliminated.

Blom and Treschow (6) mention that organic matter either reduces the nitric acid or in other ways interferes in the nitration process. Alten and Weiland (1) arrived at the conclusion that organic matter which, on the addition of the sulphuric acid, turns brown interferes in the nitration and should not be allowed to be present. Rauterberg and Benischke (13) suggest that 40—200 mg dry matter be extracted with 0.14 N sulphuric acid and evaporated to dryness with sodium hydroxide before nitration. Treschow and Gabrielsen (15) and Alten et al. (2) prefer to use the organic material directly while Jones and Underdown (12) remove a number of interfering substances by passing the extract through an ion exchange column.

On account of the high content of sugar in dried sugar beet a considerable reduction in the quantity of organic matter present would be effected by removing the sugar. For this purpose precipitation by means of calcium, strontium, or barium (Dahlberg, 8) might be used. Precipitation with barium proved to result in satisfactory precipitation of sucrose, but at the same time a certain amount of nitrate was precipitated.

According to Alten and Weiland's investigations (1) a number of cations are not suitable as precipitating agent in an alkaline medium of water on dried pulp. Under certain conditions the copper ion is able to cause proteins to precipitate, and an experiment showed that in the filtrate from an alkaline solution of tannic acid — in which precipitation had been caused by the addition of copper sulphate — it was impossible by the addition of sodium hydroxide to reproduce the dark colour of the alkaline tannic acid solution, it was assumed that the copper ion — at any rate to a certain extent — was able to remove tannins. Furthermore, with regard to salts of oxalic acid — of which a certain amount may occur in sugar beet — the copper salt is sparingly soluble. In addition to those already mentioned there are other compounds which are precipitated out by copper ions (Feigl, 9).

Extraction experiments with different quantities of sodium hydroxide and copper sulphate showed that when 1—2.5 g dried pulp are extracted with 39 ml water and 5 ml 2 N sodium hydroxide and 6 ml of saturated copper sulphate solution is added to the extract which is shaken for half a minute before it is filtered, a clear extract will be obtained. Experiments showed that a procedure like the above did not result in any changes in the nitrate content of a sample of vegetable matter. Amounts of nitrate added were recovered. Moreover, from a few experiments it was found that no alteration

Table 3. *Experiments with extract corresponding to different quantities of dry matter per analysis.*

Sample	mg. dry matter	µg NO$_3$–N found	% NO$_3$–N in dry matter	mg dry matter	µg NO$_3$–N found	% NO$_3$–N in dry matter	NO$_3$–N found in % of calculated
e$_1$, root	79.3	134.2	0.179	19.8	34.1	0.172	101.5
e$_2$, root				20.5	35.2	0.172	
e$_3$, root	80.7	141.1	0.175	20.2	35.7	0.177	101.1
f$_1$, root	79.6	177.2	0.223	19.9	45.0	0.226	101.6
f$_2$, root	81.8	181.0	0.221	20.5	45.2	0.220	99.8
f$_3$, root	81.4	177.2	0.218	20.4	44.9	0.220	101.4
e$_1$, top	34.7	181.0	0.522	8.68	45.9	0.528	101.1
e$_2$, top	35.2	181.0	0.514	8.80	46.2	0.525	102.0
e$_3$, top	34.2	176.9	0.517	8.55	44.4	0.519	100.3
f$_1$, top	16.4	109.2	0.667	4.10	27.2	0.664	99.5
f$_2$, top	17.1	115.2	0.674	4.28	28.3	0.662	98.4
f$_3$, top	16.2	108.6	0.668	4.06	27.6	0.680	101.5
b$_{53}$, root	196	5.6	0.00285	99.8	2.90	0.00290	103.6

in the content of nitrate occurred during the drying process, whether the fresh pulp was treated with sodium hydroxide or toluene or not.

Extraction of nitrate from vegetable matter seems to occur readily (7). In the present investigations 3 ml water and 25 ml 0.2 N NaOH were added to samples of 1.4 g dried pulp of the above described nature which were shaken for different periods in a shaking apparatus before copper sulphate was added. The results of the nitrate determinations remained unchanged when the shaking period varied from 15 minutes to 4 hours.

A treatment of the extract with activated carbon did not influence the dependence of the analytical results on the quantity of dry matter used per analysis, but as it will appear from the following tables, it was found that an admixture of silver acetate to the extraction medium gave satisfactory results. For the following experiments the dried beet material (usually 400—500 mg) has been extracted with 3 ml of 0.5 N sodium hydroxide, 10 ml saturated (at 23° C) silver acetate solution and 2 ml saturated (at 23° C) copper sulphate solution. The expression "mg dry matter" in tables 3 and 4 indicates the amount of dry matter to which the amount of extract used per analysis corresponds: e$_1$, e$_2$, e$_3$ . . . . denotes different extracts prepared from the same batch of dried sugar beet pulp.

When considering the influence of the amount of dry matter used per analysis on the results obtained, it will be seen that — apart from sample b$_{53}$ in which the nitrate percentage is very low — the use of a small quantity of material per analysis (right-hand part of table 3) has produced results which are equal to 100.75 ± 0.35 % of the results found when a large quantity of material has been used per analysis (left-hand part of table 3). On the

Table 4. *Experiments on the addition of nitrate to the nitration medium.*

Sample	µg NO$_3$–N found in extract	µg NO$_3$–N added	µg NO$_3$–N found after addition of NO$_3$	µg NO$_2$–N found in % of expected quantity
a$_1$ root	26.1	40	67.3	101.8
a$_2$ root	26.0	40	66.2	100.4
b root	29.9	40	70.2	100.4
c$_1$ root	98.8	40	140.3	101.1
c$_2$ root	100.8	40	139.8	99.4
d$_1$ root	110.4	40	149.0	99.2
d$_2$ root	107.5	60	169.0	100.9
d$_3$ root	94.4	60	157.5	101.9
a$_1$ top	41.7	40	82.1	100.5
a$_2$ top	41.9	40	82.0	100.1
b top	52.0	40	92.8	100.5
c$_1$ top	140.5	40	181.5	100.6
c$_2$ top	141.7	40	182.0	100.2
d$_1$ top	132.6	40	176.4	102.2
d$_2$ top	131.0	40	172.8	101.1
b$_{53}$ root (100 mg)	2.90	10	13.4	103.7

basis of this material, which comprises 30 nitrate determinations, it may be concluded that the probability that any difference should exist is very small.

Table 4 lists results from experiments in which potassium nitrate has been added to the nitration medium. Extracts corresponding to about 80 mg dry matter from roots and about 35 mg dry matter from tops have been used.

The results given in table 4 show that after addition of potassium nitrate to extracts of different origin and prepared from materials with different contents of nitrate, analytical results have been obtained which equal 100.69 ± 0.48 % of the calculated content of nitrate-N. The results obtained were identical whether potassium nitrate was added to the material before or after extraction.

For the purpose of comparison with the results given in tables 3 and 4 table 5 summarizes a number of results obtained according to the method (15) previously referred to. For the calculation of the expression "µg NO$_3$-N found in % of the calculated quantity" in table 5 the content of nitrate in samples No. 3, 4, 9, and 10 has been designated as 100 %, and the remaining values calculated on this basis. The last column gives nitrate determinations obtained from the vegetable materials concerned when using the method devised in the present work.

The figures show that the results of the nitrate determinations — in the case of dried material — decrease considerably with increasing quantity of dry matter used per analysis, and further that added nitrate is not fully recovered. The great difference between the nitrate determinations according to the two methods is, with regard to the material consisting of tops, presumably caused by the chloride present in the sample.

Table 5. *Comparison between previous method (15) and the modified method resulting from the present work.*

| Nature of sample | No. | Results according to previous method (15) | | | | | % NO$_3$–N found acc. to new method |
		mg dry matter	µg NO$_3$–N added	µg NO$_3$–N found	µg NO$_3$–N found in % of calculated	% NO$_3$–N found in dry matter	
root	1	25.5		8.0	104.1	0.0275	0.0319
	2	24.1		5.7			
	3	52.3		13.5	100	0.0264	
	4	48.2		13.0			
	5	47.8	40	44.5	87.3		
	6	48.4	40	47.5			
	7	95.0		22.8	87.5	0.0231	
	8	102.4		23.0			
top	9	25.2		40.2	100	0.161	0.528
	10	25.6		41.5			
	11	25.0	40	76.0	96.7		
	12	25.5	40	80.0			
	13	50.4		79.0	98.0	0.158	
	14	50.4		80.0			
root 53 b	15	24.9		1.0	100	0.0020	0.0029
	16	25.0		0			
	17	49.8		0.8	88.5	0.0018	
	18	50.4		1.1			

Standard curves. Standard curves were plotted for analytical materials with increasing contents of potassium nitrate, the analytical sample used being 70 mg of sucrose per analysis. The results used for the standard curve must be obtained under the same conditions as those prevailing during the determinations of nitrate in vegetable matter.

If, *e.g.*, it is desired to dilute the distillate to a volume of 400 ml, the distillates for the standard curve must likewise be diluted to 400 ml as the results obtained will otherwise be too low. These experiments confirm the data obtained by Rauterberg and Benischke (13).

This may possibly be accounted for by decrease in the nitroxylenol yield with increasing quantity of nitrate present. However, solutions of different concentrations of pure nitroxylenol also exhibited a bend in the curve when measured on the colorimeter. Since different batches of xylenol may produce different nitroxylenol yields, allowance must also be made for this fact in the plotting of the standard curve.

Discussion. If the time used for the extraction with sodium hydroxide and silver acetate is increased considerably beyond the time prescribed in the analytical method given in the present work, the nitroxylenol yield will increase, presumably on account of oxidation of other nitrogen compounds. Extracts which had been filtered on the other hand proved to be stable for at least one week.

Table 6. *Standard deviations in analytical results.*

μg NO_3–N per analysis	Standard deviation when making			
	number of determ.	2 determ. on the same extract	number of determ.	1 determ. on each of 2 extracts
< 10	6	1.3 %	8	4.1 %
10— 50	12	0.66 %	14	1.09 %
50—200	30	0.82 %	18	1.05 %

During the nitrate determinations errors of different kinds may be introduced.

When the standard deviation, s, of the analytical results was determined according to the formula: $s^2 = \dfrac{(d^2)}{2p}$, where d is the difference between two duplicate determinations and p is the number of samples, the results in table 6 were obtained:

Within the group "< 10 μg nitrate-N per analysis" the error due to the extraction seems to be considerable, which may probably be accounted for by the fact that the ratio of dry matter to liquid had to be made large to obtain an extract which was sufficiently concentrated. The quantities of nitrate present within this group have varied between 1.3 and 5.9 μg nitrate-N per analysis.

Decomposition and distillation of nitroxylenol was the same whether pure nitroxylenol was added to a nitration medium containing dried sugar beet pulp or potassium nitrate.

The method has been used for the determination of nitrate in material from experiments fertilized with increasing quantities of nitrogen to beet-roots, and to oats, and has proved satisfactory. Furthermore it is both inexpensive and rapid.

IV. Analytical Procedure

Reagents: 75 % sulphuric acid (75 ml conc. sulphuric acid (sp. gr. 1.84) + water to produce a total volume of 100 ml). Xylenol mixture: 1.016 g 2 : 4-xylene-1-ol + 5 ml glacial acetic acid. Copper sulphate solution (saturated at room temperature), 2 N and 0.5 N sodium hydroxide, ethanol (absolute), 20 % ethanol, silver acetate solution (saturated at room temperature) and potassium nitrate.

Extraction: From finely ground vegetable material, which has been dried at 80° C, weigh out a sample of up to 500 mg in a 100 ml flask, add 3 ml of 0.5 N sodium hydroxide and 10 ml saturated silver acetate solution, shake

for half an hour in a mechanical shaking apparatus. Add 2 ml saturated copper sulphate, shake again, shortly but vigorously by hand, set aside for 5 minutes and filter through a dry filter. Draw an aliquot containing between 1.5 and 200 µg nitrate-N.

Nitrate determination: Transfer 3 ml of extract or less plus water to produce a volume of altogether 3 ml by means of a pipette into a 250 ml flat-bottomed pyrex flask. Add 0.2 ml xylenol mixture and, within a period of 15 minutes, 22 ml of 75 % sulphuric acid and stopper the flask immediately. Shake the mixture at once and again after a few minutes, imparting a vigorous swirling motion to the liquid. Set aside the flask for nitration for 40—60 minutes at a room temperature of 12—28° C, shake again, add 75 ml of tap water, and replace the stopper. A flask containing a nitration or distillation mixture must always be given a brief shaking before removing the stopper. Add half a teaspoonful of pumice and, immediately before starting the distillation, which should be commenced within a period of $^1/_2$—2 hours after the addition of tap water, add 5 ml of absolute ethanol.

The distillation apparatus consists of a Hopkins splash head, a discharge tube and a 15—20 cm long condenser with a helical passage. If ground-glass joints were not available, the individual parts were connected by means of rubber tubes and stoppers respectively. The ends of abutting faces of splash head and discharge pipe should be fixed so as to be as close to each other as possible. The discharge tube should have a slight downwards slope. A screen should be inserted between gas flame and receiver to avoid any heating of the distillate. Place the distillation flask on wire gauze with asbestos and, using a strong flame, perform the distillation in the course of about 10 minutes. Distill over 20—22 ml into an empty receiver (e.g. a 50 ml measuring cylinder), remove the distillation flask and flush the condenser with 15 ml of 20 % ethanol. Transfer the distillate quantitatively into a 100 ml or a 50 ml measuring flask and replace its stopper. If the analytical sample contains less than 40 µg nitrate-N, the colorimetric measurement should be made within a period of four hours, if the nitrate content exceeds 40 µg, the distillates may be left for 24 hours. Immediately before making the colorimetric measurement, which is made at λ 460 mµ, add 3 ml of 2 N sodium hydroxide and dilute to the mark with water. The density read is referred to a standard curve from which the nitrate content in the sample may be read.

The standard curve is plotted on the basis of determinations of nitrate in potassium nitrate solutions with known contents of nitrate. A sample of 3 ml of the nitrate solution is used per analysis instead of extract. "Extraction" of potassium nitrate is not necessary, but otherwise analyses for the plotting of standard curves and for the determination of nitrate in

vegetable material must be made according to absolutely identical proce-
dures. Instead of dried vegetable matter about 70 mg of sucrose is added
per analysis.

Distillation of a mixture of water and sulphuric acid should be made
daily and the density of the distillate should be measured to ensure that the
apparatus is clean as the rubber material used for connecting the individual
parts of the distillation apparatus in the course of time may alter with regard
to its tendency to give off light-absorbing substances; different rubber
qualities differ with regard to this condition.

Summary

The xylenol method for determination of nitrate in plants was investigated.
The presence of limited quantities of copper and silver ions and of tyrosine,
glutamic acid, asparagine, betaine, and ammonium ions in the quantities
which may occur in vegetable material does not interfere with the nitrate
determination.

Addition of ethanol before distillation prevents oxidation of xylenol in the
distillate and keeps the condenser clean.

Fractional distillation of blanks as well as of analytical samples show that
the rate of distillation of side products was greater, the longer the distillation
proceeded.

Extraction of nitrate from dried beet root and beet tops by means of an
alkaline medium containing silver acetate and subsequent precipitation with
copper sulphate resulted in an extract of suitable nature for the nitrate
determination.

The analytical error has been found to be less than 1.1 % when deter-
mining quantities of nitrate-N amounting to 10—200 µg. The method has
been tried within the range 1.3—200 µg nitrate-N.

The investigation has shown that the xylenol method devised by Treschow
and Gabrielsen when subjected to certain modifications can also be used
successfully for the determination of nitrate in beet roots and beet tops.

References

1. Alten, F. & Weiland, H.: Vergleichende Untersuchungen über die kolorimetrischen
   Methoden der Nitrat-Bestimmung. — Z. Pflanzenern., Düng., Bodenk. A 32: 337.
   1933.
2. Alten, F., Wandrowsky, B. & Hille, E.: Die Bestimmung des Nitratstickstoffes in Pflan-
   zensubstanzen als Nitroxylenol. — Bodenk. u. Pflanzenern. 1: 340. 1936.

3. Andreotti, R.: Determination of nitrates and nitrites in salted meat. — Ind. ital. conserve. 27: 55. 1952.

4. Balks, R. & Reekers, I.: Determination of nitrate in plant substances. — Landw. Forsch. 6: 121. 1954.

5. Barnes, H.: A modified 2 : 4-xylenol method for nitrate estimations. — Analyst. 75: 388. 1950.

6. Blom, J. & Treschow, C.: Eine neue Methode zur quantitativen Bestimmung kleinster Mengen von Nitraten in Böden und Pflanzen. — Z. Pflanzenernähr., Düng., Bodenk. A 13: 159. 1929.

7. Burström, H.: The colorimetric determination of nitrate in plant materials. — Sv. Kem. Tidskr. 54: 139. 1942.

8. Dahlberg, H. W.: The barium process of de-sugarizing beet molasses. — Transactions of the American Institute of Chemical Engineers. 16: 35. 1924.

9. Feigl, F.: Specific and special Reactions. — Nordeman Publishing Co. Inc. New York, 1940.

10. Hamy, A.: Notes De Chimie Analytique. 1. Dosage Des Nitrates dans les Extraits Végétaux. — Ann. Agron., 15: 126. 1945.

11. Holler, A. C. & Huch, R. U.: Colorimetric determination of nitrates and nitric acid esters. — Anal. Chem. 21: 1385. 1949.

12. Jones, G. B. & Underdown, R. E.: Determination of nitrate in plant materials. — Anal. Chem. 25: 806. 1953.

13. Rauterberg, E. & Benischke, H.: Determination of nitrate as nitroxylenol in crops. — Bodenk. u. Pflanzenern. 31: 216. 1943.

14. Snell, F. D. & Snell, C. T.: Colorimetric Methods of Analysis II. — 3rd Ed. D. Van Nostrand Co., Inc., New York. 1949.

15. Treschow, C. & Gabrielsen, E. K.: Zur Bestimmung von Nitrat in Pflanzen und Böden. — Z. Pflanzenernähr., Düng., Bodenk. A 32: 357. 1933.

16. Yagoda, H. & Goldman, F. H.: Analyses of atmospheric contaminants containing nitrate groupings. — J. Ind. Hyg. Toxicol. 25: 440. 1943.

Catalysis of Indoleacetic Acid Oxidation by Manganic Ions

By

G. A. Maclachlan and E. R. Waygood

Department of Botany, University of Manitoba,
Winnipeg, Canada
(Received March 14, 1956)

Introduction

Recently, Waygood *et al.* (22) have described a thermolabile enzyme preparation from wheat leaves that catalyses the complete decarboxylation and oxidation of indoleacetic acid at pH 6.0 and which depends for full activity on the concomitant production of manganic ions. Trivalent manganese was continuously generated by a manganese-phenol-peroxidase system (Kenten and Mann, 13) and hydrogen peroxide produced during the oxidation (Andreae and Andreae, 2, Waygood *et al.*, 22). The reaction is autocatalytic and completely inhibited by either citrate or pyrophosphate, both of which strongly chelate manganic ions. Dependence of the system on peroxidase could account for its thermolability, but since the wheat leaf extracts were not clarified beyond dialysis, it was possible that some of the steps of the oxidation were also enzymically catalysed. In particular, because blue light and oxygen overcame the lag period by accelerating the production of hydrogen peroxide, the presence of a specific flavoprotein component that had been postulated earlier (Galston *et al.*, 8) was tentatively accepted as a possibility.

In order to isolate the reaction between indoleacetic acid and manganic ions a method was required for generating the ions non-enzymically at physiological pH. This paper describes such a method which utilizes the complex formed by manganic ions and ethylenediaminetetracetate (Přibil and Horáček, 19), hereafter referred to as versene.

21

Experimental Results

1. *The effect of manganic complexes*

Because of its extremely high redox potential ($E_0 = 1.51$ v) the manganic ion can have only a transient existence at neutral pH unless stabilised in complex with an anion that does not undergo oxidation e.g. pyrophosphate or citrate. The stability of manganipyrophosphate and manganicitrate implies that little ionization occurs and this is reflected in their low redox potentials at pH 6.0 ($E_0' = $ ca. 0.60 v) and in the inhibition by citrate and pyrophosphate of the enzymically catalysed oxidation of indoleacetic acid (Waygood *et al.*, 22, Kenten, 12). On the other hand the complex formed by manganic ions with versene is relatively unstable (Přibil and Horáček, 19). Since we found that versene did not inhibit, but enhanced the rate of enzymic oxidation of indoleacetic acid, the reaction between manganiversene and indoleacetic acid was examined.

Manganiversene was prepared by vigorously shaking two volumes of 0.1 M versene, pH 6.0 and one volume of 0.1 M manganous chloride with either solid lead dioxide or manganese dioxide as oxidant according to the following equation (Přibil and Horáček, 19).

$$2(MnV)'' + Pb^{\cdots\cdot} \rightarrow 2(MnV)' + Pb^{\cdot\cdot}$$
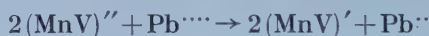
Manganous ions replace hydrogen ions in the versene molecule thereby resulting in a lowering of pH to about 3.5. The pH increases to 4.0—4.5 during oxidation to the manganiversene complex which is coloured an intense red (λ max. $= 500$ mµ). Above pH 6.0 the colour reversibly changes to yellow. In the transition range pH 5.0 to 6.0 the complex is least stable and decolourizes in approximately one hour as compared to 12 hours at pH 4.0.

When indoleacetic acid was added to manganiversene at a pH of 5.0 or lower an immediate colour change occurred from red through purple to colourless. This was accompanied by the formation of a voluminous purple-black precipitate. The final colourless supernatant liquid gave no reaction for indoleacetic acid with the Salkowski reagent, although a weak reaction with the washed precipitate indicated that some indoleacetic acid, at least in hydrolysable form, was still present. Above pH 5.0, manganiversene solutions were also decolourized by indoleacetic acid, but in contrast a thermostable yellow product (Salkowski-negative) formed in solution without any visible precipitation.

The behaviour of indoleacetic acid under these conditions is probably the result of a direct reaction with manganic ions and not with hydroxyl radicals or hydrogen peroxide which might be expected to form by the reaction of

Table 1. *Destruction of indoleacetic acid by low concentrations of manganiversene.* See text for experimental conditions and concentration of reactants in the non-enzymic system; wheat leaf extract prepared according to Waygood *et al.* (22) and 0.5 ml. used; molar equivalence of gas exchanged=158 μl.

System	% Destruction (Salkowski test)	Carbon dioxide released (μl)	Oxygen consumed (μl)
manganiversene	96.0	158.5	149.5
manganiversene plus wheat leaf extract	92.0	143.0	133.5

manganic ions with water (Bawn and White, 5). The latter reaction is unlikely since significant amounts of oxygen release could not be detected during the spontaneous decolourization of manganiversene. Furthermore, the redox potential of aerated manganiversene solutions at room temperature titrated to 50 per cent oxidation with ferrous ions was found to approach ($E_o' = 0.710$ v at pH 6.2 and $E_o' = 0.807$ v at pH 4.2) but never exceeded that of the water/oxygen potential ($E_o' = 0.821$ v at pH 6.0). It is significant that the potentials of solutions of manganicitrate and manganipyrophosphate attained this magnitude only below ca. pH 4.0. Accordingly in this pH range these complexes were decolourized rapidly by indoleacetic acid with the formation of a purple-black precipitate typical of the manganiversene reaction whereas above pH 4.0 no precipitation took place. Cobaltiversene and ferriversene, having redox potentials below 0.5 v between pH 4.0 and pH 6.0 were unable to react with indoleacetic acid. These observations suggest that a potential of about 0.7 v is required before indoleacetic acid can be oxidised. The hydrogen peroxide/oxygen potential ($E_o = 0.682$ v) is very close and would account for the reported direct oxidation of indoleacetic acid by hydrogen peroxide and peroxidase systems (Goldacre, 10, Waygood *et al.*, 22).

2. *Gaseous exchange*

The reaction between manganiversene and indoleacetic acid was investigated manometrically with conventional Warburg respirometers and flasks of volume ca. 20 ml. held shaking in a bath at 28.6° C. In order to duplicate conditions for the enzymic reaction (Waygood *et al.*, 22) as closely as possible, 6.6 μM of indoleacetic acid (sodium salt, pH 6.0) was added to a system buffered with veronal or 150 μM orthophosphate (pH 6.0) containing 3.0 μM versene, 3.0 μM manganese chloride and 30 μM manganese dioxide in a final volume of 3.0 ml. The presence of excess manganese dioxide permitted continuous generation of the mangani-complex at a maximum concentration of 10^{-3} M. Under these conditions the addition of indoleacetic acid resulted in the release of close to one mole of carbon dioxide and the consumption of one mole of oxygen per mole of indoleacetic acid (Table 1). Gas exchange
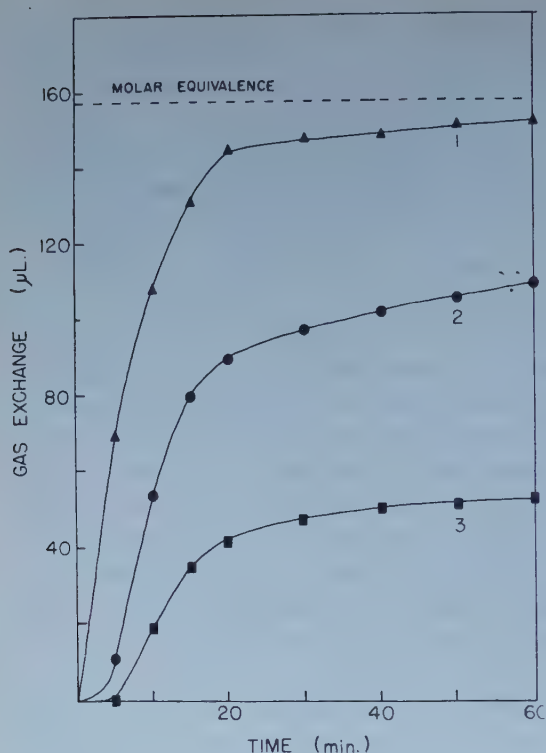
Figure 1. *Gaseous exchange of the reaction between indoleacetic acid and manganiversene.* Reaction initiated by adding 6.6 μM indoleacetic acid to a system containing 1.5 ml. veronal buffer pH 6.0, 30 μM manganese dioxide, 15 μM versene, 15 μM manganous chloride, total volume 3.0 ml.

Curve 1. Carbon dioxide release in air
Curve 2. Oxygen consumption in an oxygen atmosphere
Curve 3. Oxygen consumption in air

was slow as compared to the enzymic reaction and attained complete equilibrium in 180 min., after which the Salkowski test confirmed the disappearance of indoleacetic acid. Well dialysed extracts of wheat leaves slightly depressed the rate of oxidation, the final equilibrium, and the extent of indoleacetic acid oxidation (Table 1). The systems were unaffected by light or catalase. It is concluded that if these extracts contain enzymes which catalyse one or more of the steps in the reaction sequence they are not operating prior to the reaction with manganic ions nor do they appear to be essential for decarboxylation or oxygen consumption.

When indoleacetic acid was added to a more concentrated manganiversene solution *i.e.* 5×10^{-3} M (using veronal buffer and manganese dioxide in this instance), carbon dioxide release was almost complete within 20 min. and approached the theoretical molar equivalence in an atmosphere of air. However, oxygen consumption was depressed to about one third of a mole per mole of indoleacetic acid and began only after a definite lag period. The lag was diminished and oxygen uptake was increased when an atmosphere of oxygen replaced air. (Figure 1). The final solutions were coloured yellow

and several tests showed close correlation between the amounts of carbon dioxide released and indoleacetic acid destroyed, although these varied between 85 per cent and 100 per cent.

Anaerobically, in a nitrogen atmosphere with the same concentration of reactants, carbon dioxide was released in amounts ranging from 55 per cent to 90 per cent in different experiments. The colour of the reaction mixture changed to purple and a precipitate was formed which appeared to be identical with that formed aerobically below pH 5.0.

The reactions in air, oxygen, and nitrogen were repeated in systems containing maganiversene, but from which manganese dioxide or lead dioxide was removed by centrifugation. The results essentially duplicated those in the presence of oxidant. Complete decarboxylation and the uptake of one mole of oxygen was attained only in an oxygen atmosphere and when the molar concentration of manganic ions was initially equal to or greater than that of indoleacetic acid.

3. *Reactions of related compounds*

The reactions of manganiversene with some indole compounds and other substances related in structure and biological effect are summarised in Table 2. Gas exchange of the reactions was measured at pH 6.0 under the same conditions as those described for Figure 1 with the exception that in some cases lead dioxide and orthophosphate were used instead of manganese dioxide and veronal buffer. The reaction of systems brought to pH 4.0 with acetic acid and containing 16.5 μM of substrate and a maximum of 165 μM of manganiversene (oxidant removed) was followed qualitatively with reference to colour change, precipitation or polymerization, and rate of decolourization of manganiversene.

All of the indole compounds tested with the exception of tryptophane consumed oxygen at pH 6.0. The overall oxygen consumption approached equilibrium in one hour and was invariably increased by using an atmosphere of oxygen in place of air. Only indoleacetic and indolepropionic acids released carbon dioxide in significant amounts. The non-indole growth regulating substances consumed little oxygen and no more than one quarter of the total carbon dioxide theoretically available in their carboxyl groups was released.

In the reaction at pH 4.0 all of the indole compounds decolourized manganiversene at room temperature in less than one hour and all yielded a distinctively coloured precipitate or solution with the exception of acetyltryptophane. The fact that these substances can decolourize ten times their concentration of manganiversene indicates the initiation of a chain reaction involving polymerization. There was no apparent reduction of mangani-

Table 2. *Reaction of various indoles and growth-regulating substances with high con-centrations of manganiversene.* See text for concentration of reactants; molar equivalence of gas exchanged=158 µl.

Substrate	Reaction pH 4.0	Gas exchanged, pH 6.0 (µl)			
		Carbon dioxide released		Oxygen consumed	
		Air	Oxygen	Air	Oxygen
Indole	bright orange ppt.	—	0	60.0	90.0
Skatole	pink ppt.	—	0	78.5	96.5
Indoleacetic acid	purple-black ppt.	153.0	135.0	53.0	111.0
Indolepropionic acid	yellow ppt.	20.5	85.5	69.0	147.0
Indolebutyric acid	green-black ppt.	15.0	29.0	37.0	93.0
Tryptophane	yellow solution	—	34.0	—	0
Acetyltryptophane	rapid decolourization	—	40.0	—	26.0
2,4-Dichlorophenoxyacetic acid	none	—	15.5	—	9.0
2,4,5-Trichlorophenoxyacetic acid	none	—	32.0	—	9.5
Naphthylacetic acid	none (insoluble)	11.0	40.5	4.5	42.5
Naphthoxyacetic acid	none	—	26.5	—	2.5

versene by the nonindole compounds and neither did these produce charac-teristically coloured solutions.

In general, indoleacetic acid was attacked more vigorously by mangani-versene at pH 6.0 than were other indole compounds. However it is probable that by changing the pH their rate of destruction as measured by oxygen uptake could be increased. In one experiment, skatole was found to absorb rapidly one equivalent of oxygen at pH 7.0 as compared to a slower absorp-tion of approximately one half equivalent at pH 6.0.

Discussion

The above data show that the manganic ion reacts with certain indoles to form a product which, in some cases, is capable of consuming oxygen spon-taneously. The amount of oxygen consumed at pH 6.0 appears to depend on the nature of the side chain although the presence of a side chain is not essential since indole itself is rapidly oxidized. The reactions described suggest that an unstable indole-manganic complex is formed with points of attachment at the nitrogen group and, if present, the carboxyl group. With respect to indoleacetic acid, the intermediate product of this chelation is probably a free radical since the final product may be a precipitate (poly-mer) of varied composition or a soluble substance formed by reaction with oxygen. The conditions necessary for maximum oxygen uptake i.e. low con-centration of manganic ions or increased oxygen tension, both tend to decrease the concentration ratio manganic/oxygen, suggesting that poly-

merization of the free radical intermediate is a much faster process than its aerobic oxidation. Oxygen is well known to be an effective retarder of polymerization by combination with free radicals (Bacon, 4) and it is also possible that manganic ions when in excess, replace oxygen as oxidant of the free radical intermediate. While the polymerization product and the soluble yellow product in oxygen have not been identified as yet, the latter appears to be chromatograph'cally identical to the product of enzymic degradation which is also yell w coloured Both are non-Salkowski reacting. but both give a pink colouration with concentrated hydrochloric acid indicat've of pyrrole and some indole structures.

The ques ion of whether hydrogen peroxide is produced from indoleacetic acid in he non-enzymi reaction cannot be answered unequivocally until more is known about the nature of the end products. It may be noted that molar equ'valences of oxygen uptake were attained in the presence of excess manganiversene, manganese dioxide, or catalase, all of which are capable of releasing oxygen from hydrogen peroxide stoichiometrically or catalytically. If hydrogen peroxide is produced and also decomposed then the real molar oxygen equivalence would exceed unity.

Catalysis of several organic oxidations by manganese has been known for some time. Kagan and Lubarsky (11) showed that acetaldehyde autoxidation is accelerated by manganous ions due to their reversible oxidation and redu - tion by the inte mediate peracetic acid. The manganese catalysed oxidations of oxalacetate o malonate (Vennesland and Evans, 21) and several dicarboxylic acids including dioxymaleic acid by a manganese-phenol-peroxidase system (Kent n and Mann 14) can be explained satisfactorily only by assuming reversible oxidation of manganese.

The mechanism of these reactions may be similar in many respects to that proposed by Bawn and White (6, 7) for the cobaltic ion-catalysed oxidation of s mple organic acids, aldehydes and alcohols. From kinetic studi s these authors concluded that the cobaltic ion abstracts an electron and a hydrogen ion from each substrate leaving a highly reactive free adical. In the case of the manganic-catalysed oxidation of indoleacetic acid the primary reaction must involve the carboxyl group, since carbon diox de is liberated both aerobcally and anaerobically and precedes oxygen uptake (Figure 1, Waygood et al., 2?). An analogous reaction takes place in the explosi e decarboxylation and polymerization of furoperacid (Milas and McAlevy, 18). If the manganic ion, by placing a high positive charge on the carboxyl group of indoleacetic acid withdraws an electron and a hydrogen ion, the release of arbon dioxide would result in a 3-methyl-indole radical Skatole is actively oxidized by manganiversene (Table 2) and by this mechanism would yield the same radical. This could conceivable react with water, itself. another

radical, indoleacetic acid, manganic ions or oxygen. The actual fate of the radical would depend on the rate constants of the possible reactions involving the formation of either hydroxyl radicals (and therefore hydrogen peroxide), a dimer or polymer, or an oxidised monomer. Competition for the radical would result in more than one reaction product which may explain the difficulties that investigators have had in identifying the end product of the enzymically catalysed reaction (cf. Manning and Galston, 17).

The destruction of indoleacetic acid by manganic ions raises the question of the physiological significance of the mangano-manganic equilibrium in the growth processes of plants. Such an equilibrium is known to exist in soil (Sherman and Harmer, 20. and is due probably to the action of bacteria or fungi (MacLachlan, 16). Under certain conditions manganese dioxide has been observed to accumulate in plant tissues (Arens, 3, Kenten and Mann, 15) and presumably this oxidation *in vivo* is catalysed by an endogenous phenol-peroxidase system operating in the same way as that described by Kenten and Mann (13). The finding of such a phenol in wheat leaves (Waygood *et al.*, 22) holds special interest since these substances may be regarded as natural chelating agents as well as potential oxidants of manganese.

Among many factors which could displace this equilibrium, light through its peroxigenic effects is one of the most important. Gerretson (9) has presented potentiometric evidence for the oxidation of manganese by illuminated chloroplasts and Kenten and Mann (15) have demonstrated that manganic ions are formed by such preparations. Furthermore, Andreae (1) has found that manganipyrophosphate can be generated non-enzymically by monohydric phenols and light-activated riboflavin. Since the non-enzymic manganic-catalysed oxidation of indoleacetic acid is independent of light, the effect of light on the enzymically catalyzed reaction may be due to the accelerated production of manganic ions. Therefore there appears to be no necessity for postulating a specific flavoprotein component to catalyse the aerobic step of the oxidation, although there are many examples of spontaneous reactions that are enzymically catalysed *in vivo* and this question must remain unanswered for the time being.

The results of this investigation support the argument expressed previously (Waygood *et al.*, 22) that manganic ions initiate the oxidation of indoleacetic acid by phenol-peroxidase systems. If manganese is oxidised by the Kenten-Mann system (13) *in vivo* the role of the mangano-manganic equilibrium and peroxidase and certain phenolic compounds would be established in the growth processes of plants. The special complexing ability of versene for manganic ions recommends it as an experimental model for simulating enzyme action and the mechanism of oxidation by manganic ions may lead also

to a clearer understanding of the role of free radicals in enzymic catalysis and oxygen activation.

Summary

Reactions between indoleacetic acid and manganiversene solutions have been studied in order to determine whether decarboxylation and oxidation of this plant hormone by unpurified manganic ion-producing enzyme systems could be duplicated non-enzymically. The results show that manganiversene catalyses the degradation of indoleacetic acid by different pathways at pH 4.0 and at pH 6.0. At the higher pH the gaseous exchange duplicates that occurring in the enzymic reaction and the products of both reactions appear to be identical. This obviates the necessity for postulating a more complex system of catalysts and cofactors in the enzymic oxidation other than those required for the formation of manganic ions.

From the reactions of indoleacetic acid, other indoles and certain growth-regulating substances at high oxygen tension and high concentrations of manganiversene it is suggested that reaction with the manganic ion produces intermediate free radicals.

The possible physiological significance of a mangano-manganic equilibrium on the growth processes of higher plants is stressed.

References

1. Andreae, W. A.: The photoinduced oxidation of manganous ions. — Arch. Biochem. and Biophys. 55: 584. 1955.
2. Andreae, W. A. & Andreae, S. R.: Studies on indoleacetic acid metabolism I. The effect of methyl umbelliferone, maleic hydrazide and 2,4-D on indoleacetic acid oxidation. — Can. J. Botany 31: 426. 1953.
3. Arens, K.: Physiologisch polarisierter Massenaustausch und Photosynthese bei submersen Wasserpflanzen. — Planta 20: 621. 1933.
4. Bacon, R. G. R.: The initiation of polymerization processes by redox catalysts. — Quart. Rev. Lond. Chem. Soc. 9: 287. 1955.
5. Bawn, C. E. H. & White, A. G.: Reactions of the cobaltic ion I. The reaction of the cobaltic ion with water. — J. Chem. Soc. p. 331. 1951.
6. — — Reactions of the cobaltic ion II. The kinetics of the reaction of the cobaltic ion with formic acid. — J. Chem. Soc. p. 339. 1951.
7. — — Reactions of the cobaltic ion III. The kinetics of the reactions of the cobaltic ion with aldehydes and alcohols. — J. Chem. Soc. p. 343. 1951.

8. Galston, A. W. Bonner, J. & Baker, R. S : Flavoprotei and perox.dase as components of the indoleacetic acid oxidase system of peas. — Arch. Biochem. and Biophys. 42: 456 1953

9 Gerretson, F C.: Manganese in relation to photosynthesis II. Redox potentials of illuminated crude chloroplast suspensions. — Plant and Soil 2 159 1950

10 Goldacre, P. L : Hydrogen peroxide in the enzymatic oxidation of heteroauxin. — Aust J. Sci. Res. B. 4 293. 1951.

11 Kagan, M. J & Lubarsky, G. D.: The intermedia e stages of aldehyd oxidation I The catalyt'c action of manganese catalyst in the various stages of the process of acetaldehyde oxidation — J. Phys. Chem. 39: 837. 1935.

12. Kenten, R. H.· The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems — Biochem. J. 59: 110. 1955.

13. — & Mann, P. J. G.: The oxidation of manganese by peroxidase systems. — Biochem. J. 46: 67. 1950.

14. — — The oxidation of certain dicarboxylic acids by peroxidase systems in presence of manganese. — Biochem J. 53: 498. 1953.

15. — — The oxidation of manganese by illuminated chloroplast preparations. — Bio chem J. 61 279 1955.

16. MacLachlan, J D.: Manganese deficiency in soils and crops I. Control in oats by spraying; studies on the ole of soil microorganisms — Sci. Agric. 22: 201. 1941.

17. Manning, D. T. & Galston, A. W.: On the nature of the enzym'cally catalysed oxidation products of indoleacetic acid — Plant Physiol. 30: 225. 1955.

18 Milas, N. A. & McAlevy, A.: Stud es in organic peroxides IV. The spontaneous decomposition of furoperacid — J Am. Chem. Soc 56: 1221. 193 .

19 Přibil, R & Horáček, J.: Use of complexones in chemical ana ysis IV. Reductometric determination of trivalent manganese. — Coll. Czech. Chem. Communs. 14: 626. 1949

20. Sherman, G. D. & Harmer, P. M.· The manganous-manganic equilibrium of soils. — Soil Sci. Soc. Am., Proc. 7: 398. 1942.

21. Vennesland, B. & Evans, E. A.: The formation of malonic acid from oxalacetic acid by pig heart preparations. — J. Biol. Chem. 156. 783. 194i

22. Waygood, E. R., Oaks, A. & Maclachlan, G. A.· On the mechanism of indoleacetic acid oxidation by wheat leaf enzymes. — Can. J. Botany 34: 54. 1956.

The Response of *Chamaenerion angustifolium* (L.) Scop. to Different Nitrogen Sources in Water Culture

By

CARL OLOF TAMM

Forest Research Institute of Sweden, Stockholm 51
(Received April 19, 1956)

In his studies of nitrate formation in forest soils Hesselman (1917) tested the leaves of a large number of forest plants for their contents of nitrate. The diphenylamine test always gave a positive reaction on leaves from young individuals of *Chamaenerion* (*Epilobium*) *angustifolium,* and older ones also very often contained nitrate. Similar results were obtained with some other species *e.g*, *Rubus idaeus* and *Rumex acetosella*), which also occur abundantly in clear-fellings burnt areas, and other habitats where nitrate is formed in the soil. Hesselman concluded that these species — or at least young and vigorous individuals — could be considered as indicators of nitrate formation in the soil Their establishment was thought to require the presence of nitrate although Hesselman remarked that they may be able to persist where nitrate is no longer formed.

Physiological experiments have been carried out by Olsen (1921) and Marthaler (1937) in order to decide whether *Chamaenerion* can use both nitrate and ammonia as sources of nitrogen. Olsen found much better growth on nitrate in sand cultures, but ascribes the poor result with ammonia to the acidification of the substrate. Marthaler obtained no growth with ammonia in water culture, and claimed that he had controlled acidity. Yet the poor growth obtained even with nitrate makes his conclusions less convincing. The lack of aeration (or stirring) may partly account for the results.

In the years 1947 and 1948 the present writer then working at the Bota-

Figure 1. *Specimens of Chamaenerion angustifolium growing in water culture with different sources of nitrogen.*

nical Laboratory of the University of Lund, experimented with *Chamaenerion angustifolium* in water cultures with different sources of nitrogen. The publication of these experiments has been delayed for a long time, awaiting the results of chemical analysis of some of the experimental material.

The first experiments were carried out with a nutrient solution suitable for wheat plants and also tested on Chamaenerion seedlings (Burström, personal communication). The basic solution contained in mmols per litre: 1 KH_2PO_4, 1 $MgSO_4$, 0.1 ferric citrate, and 0.05 $MnSO_4$. To this was added 1 mgatom N as calcium nitrate, ammonium nitrate, or ammonium chloride; in the two latter cases 0.5 calcium chloride was added. The distilled water used had been filtered through Amberlite ion exchange resin to remove traces of copper. These experiments corroborated the conclusions of Olsen (1921), that as a rule Chamaenerion grows best with ammonium nitrate, and that the poor result with ammonium chloride could be explained by acidification. Yet there were large differences between individual plants, and the best specimens from each treatment were fairly similar (Figure 1). And in the ammonium nitrate solutions there were also rather low hydrogen

ion concentrations at the end of each period (the solutions were changed weekly). In addition, the Chamaenerion leaves in these experiments contained more nitrogen than leaves collected in natural habitats, even when amply supplied with nitrogen. The leaves from plants treated with ammonium nitrate and calcium nitrate also contained much nitrate, in one experiment 0.7 and 1.2 per cent of the dry weight respectively.

Possible physiological preferences of plant species for certain sources of nitrogen are best investigated under conditions in which nitrogen acts as a limiting factor. Therefore a new experiment was carried out, which is described here in more detail.

Experimental Part

Chamaenerion seeds were sown in Petri-dishes on August 20. 1948. and planted in quartz sand on August 29. They were wate ed with a solution containing per litre 0.5 mmols of KH_2PO_4, $MgSO_4$ and NH_4NO_3, 0.1 ferric citrate, 0.05 $MnSO_4$, and $CaSO_4$ (almost saturated at room temperature). On October 11 four plants were placed in each of 12 one litre Pyrex beakers filled with nutrient solution and aerated by an air stream. These were first put in a photothermostat at $20°$, with a sodium lamp burning from 4 a.m. to 9 p.m., and later (on October 23) in a small greenhouse shaded by large trees, but with extra light (a so-called blended light lamp, Philips M.L.-500). Some of the Chamaenerion individuals had been damaged, and in half the number of beakers one seedling of *Deschampsia flexuosa* was planted instead of one Chamaenerion. The Deschampsia seedlings, however, grew very slowly in all treatments, and have been excluded from the yield figures.

From October 25 onward all beakers received the same basic solution, containing KH_2PO_4, $MgSO_4$. ferric citrate and $MnSO_4$ in the concentrations mentioned above, and in addition 0.5 mmol/l K_2SO_4. The nutrient solution was saturated with $CaSO_4$ to 7/10. The beakers were then divided in three groups, A. B. and C. The groups were made as similar as possible, though the size of the individual plants varied a great deal (in most cases between 0.2 and 0.5 g fresh weight). The beakers were arranged in a Latin square. Nitrogen solutions were added on four occasions; and some phosphorus was also given on certain occasions to counteract pH changes in the solutions. Thus on October 26 each beaker marked A received 3.5 mg N as sodium nitrate together with 7.8 mg P as phosphoric acid; beakers B 3.5 mg N as ammonium nitrate together with 7.8 mg P as NaH_2PO_4, and beakers C 3.5 mg N as ammonium sulphate together with 7.8 mg P as Na_2HPO_4. The beakers each contained 0.8 litres of nutrient solution. The nutrient solutions were renewed on 30.11, 8.11., and 20.11. More nitrogen was added on 30.10 (3.5 mg N. no P), 2.11 (1.4 mg N+3.1 mg P), and 20.11 (1.4 mg N+1.6 mg P).

The plants were harvested on November 27. Hydrogen ion concentrations in the solutions were determined at frequent intervals and the differences between different treatments were less than in earlier experiments. Yet figures down to about

pH 3.5 were observed in all treatmen s most often in the ammonium sulphate beakers. No root injuries due to the high acidity were observed. The pl nts appeared quite healthy, although rather s ender due to the relatively low light intens'ty, perhaps also to the low nitrogen supply A slight anthocyanin colouring of the leaves appeared when the plants had not received nitrogen for some day . Some of the plants in both nitrate and ammon'a beakers had flower buds at harvest time

The total amount of n trogen given after October 25 was 10 mg N pe beaker. About the same quant ty had been given before this date but was certainly only used to a small exten b the plants Th additions of P, K, Ca, Mg, Fe ai d Mn were identical in the diffe e it treatments Differences occur in the case of sulphate and sodium (treatment C rece ved more of both than A and B). As there was an excess of calcium si lphate in all beakers, the additional sulphat in treatment C had probably no large physiologic l effects.

Results

The results are presented n Tables 1 and 2 The contents of nitrogen, phosphorus, potassium. and calcium in leaves, stems. and roots were determined by methods described earlier (Tamm 1951). Th l aves were analyzed separately from each beaker stems and roots were mixed from all four beakers in each treatment The small amounts available did not allow duplicate determinations except in a f w cases, but the reproducibility of the methods must be considered as good (Tamm 1953) As the plants were nitrogen deficient at the end of the experiment, they had no ni rate content to interfere with the Kjeldahl analysis.

Table 1 shows that the highest yield was obtained with ammonia as the source of nitrog n the lowest figure occurred with ammonium nitrate, but the difference between both nitrate treatments is not very great. The leaf nitrogen percentages vary inversely with the yie d (Table 2 , as would be

Table 1. *Yield and nutrient uptake of Chamaenerion angust fo ium cultivated with different sources of nitrogen.* All values in mgs per four beakers. The Ca values between brackets was not directly determined but estimated u ing th Ca percentages of the other two stem samples

Parts of plants	Dry weight			Amounts of nut ien s											
				N			P			K			Ca		
	NO_3	NH_4NO_3	NH_4	NO_3	NH_4NO_3	NH_4	NO_3	NH_4NO_3	NH	NO_3	NH_4NO_3	NH_4	NO_3	NH_4NO_3	NH_4
Leaves	1,420	1,303	1,777	25.3	25.5	29.7	8.1	10.5	9.0	48.5	49.8	60 5	24 6	25.7	30.9
Stems	719	582	805	5 2	4.5	6.0	2.5	2.5	2.9	3 9	13 1	21.1	4.8	5.0	(5.7
Roots	1,296	1,189	1,791	13 2	3.8	17.2	19.7	21.2	22 0	33 2	39.9	50.6	12.7	11.5	15.9
Sum	3,435	3,074	4,373	43.7	43.8	52.9	30.3	34 2	33 9	95 6	102 8	132.2	42.1	42.2	52.5

Table 2. *Plant weights and leaf nutrient concentrations in Chamaenerion angustifolium, cultivated with different sources of nitrogen.*

Source of Nitrogen	Fresh weight Mgs/Beaker	Dry Weight Mgs/Beaker	Per Cent Dry Weight			
			N	P	K	Ca
NO_3	7,670	929	1.95	0.66	3.95	1.91
	8,250	969	1.55	0.44	3.26	1.60
	8,260	859	1.80	0.59	3.37	1.82
	5,390	678	1.87	0.62	3.02	1.69
Average	7,390	859	1.79	0.58	3.40	1.76
NH_4NO_3	7,200	758	2.05	0.73	4.14	2.15
	6,330	666	2.22	0.86	3.99	1.96
	7,150	761	1.94	0.70	3.78	1.92
	7,940	889	1.69	0.91	3.42	1.87
Average	7,160	768	1.98	0.80	3.83	1.98
NH_4	8,840	1,242	1.60	0.52	3.59	1.54
	9,410	1,233	1.49	0.38	2.78	1.86
	7,740	944	1.99	0.60	3.37	1.78
	7,920	954	1.66	0.55	4.02	1.76
Average	8,480	1,093	1.68	0.51	3.44	1.74

expected in an experiment with limited amounts of nitrogen. A similar trend can also be traced in the case of the mineral nutrients: the ammonium nitrate plants contain the highest concentrations of P, K, and Ca, at the same time they are smallest. Yet there is no strict relationship.

The amounts of nutrients taken up per four beakers are given in Table 1. The sodium nitrate and ammonium nitrate plants contained equal amounts of nitrogen, while the ammonia plants evidently had consumed more. As only 10 mg N per beaker had been added after October 25, the plants seem to have contained at least about 3 mg N per beaker on that date. As stems and roots could not be analyzed individually, it is not possible to calculate the error in the determination of the total uptake of nitrogen.

The results may be summarized as follows: 1) Good growth of nitrogen-deficient plants was obtained with all three forms of nitrogen supply. A slightly better yield was obtained with ammonium sulphate than with sodium or ammonium nitrate, which is in contrast to earlier experiences.

2) Most of the nitrogen added during the experiment — in particular the ammonia — was taken up by the plants and used for growth.

3) The source of nitrogen did not much affect the uptake of calcium, which was used roughly in proportion to growth. Phosphorus uptake was highest in the ammonium nitrate cultures. Potassium uptake was lowest in the sodium nitrate cultures, and highest in the ammonia cultures. The con-

centrations of these mineral nutrients in the plants were very high, as might be expected in a water culture experiment with nitrogen supply limiting growth.

Discussion

The experiment described here shows clearly that *Chamaenerion angusti-folium* can use both ammonia nitrogen and nitrate nitrogen. The experimental conditions were rather different from those in natural habitats of Chamaenerion, but there is hardly any doubt that the plant can also use ammonia nitrogen in nature. The present conclusions are thus inconsistent with those of Marthaler (1937), who found Chamaenerion to be an obligate nitrate plant in his experiments. On the other hand no contradiction exists between the present results and those of Olsen (1921), who ascribed his poor result with ammonium chloride to acidification of the substrate.

Regarding the ecological preferences of the species, physiological experiments of the type described here cannot supply more than a part of the information necessary. The slightly better result with ammonia nitrogen, in this experiment, may be a consequence of the experimental conditions (supraoptimal concentrations of some cations?), and nitrate nitrogen may still be better under natural conditions. There is also a difference in behaviour of the ammonium ion and the nitrate ion, which may have an ecological significance: the former is strongly absorbed by soil colloids, while the latter is not. Yet the present evidence does not suggest Chamaenerion angustifolium as a nitrate plant in the strict sense. On the other hand it certainly requires abundant nitrogen, and occurrences of vigorous Chamaenerion may therefore be taken as indicators of a good supply of nitrogen. According to Romell (1935) there is a close connection between the ammonia concentration, competition for soluble nitrogen, and nitrification in forest soils. A certain concentration of ammonia is required to maintain an active population of nitrite and nitrate bacteria, and if the ammonia is consumed by roots or mycorrhizas almost as soon as it is liberated from the organic débris, no nitrate will be produced. The occurrence of nitrate in forest soil thus signifies an ample supply of nitrogen and, in consequence, favourable conditions for Chamaenerion angustifolium. The relationship between soil nitrate and Chamaenerion establishment is probably an indirect one; yet the correlation seems to be very close, as illustrated by Hesselman's finding that young and vigorous specimens of the plant always contain nitrate in their leaves. Leaf nitrate, of course, means that nitrate occurs in the soil; there is a further prerequisite for the occurrence of nitrogen in leaves, which also appears to be fulfilled in the case of Chamaenerion: that nitrate reduction in the plant must not be too rapid (cf. Burström 1945).

Summary

After some preliminary experiments on the response of *Chamaenerion angustifolium* in water culture to different sources of nitrogen, an experiment was carried out in which only small amounts of sodium nitrate, ammonium nitrate, or ammonium sulphate, respectively, were added at intervals, in order that nitrogen supply should be the factor limiting growth. Good growth was obtained with all three sources of nitrogen, and therefore *Chamaenerion angustifolium* cannot be considered an obligate nitrate plant. An alternative explanation of the ecological preference of the species — for habitats in which nitrate formation takes place in the soil — is offered by the known connection between supply of ammonia nitrogen and other nutrients on the one hand, and soil nitrification on the other.

References

Burström, H.: The nitrate nutrition of plants. — Ann. Agr. Coll. Sweden 13: 1. 1945.

Hesselman, H., Studier över salpeterbildningen i naturliga jordmåner och dess betydelse i växtekologiskt avseende. (Summary in German). — Meddelanden Statens Skogsförsöksanst. 13—14: 297. 1917.

Marthaler, H.: Die Stickstoffernährung der Ruderalpflanzen. — Jahrb. f. wiss. Bot. 85: 76. 1937.

Olsen, C.: Studies on the hydrogen ion concentration of the soil and its significance to the vegetation. — Compt. rend. Lab. Carlsberg. 15(1): 1. 1921.

Romell, L.-G.: Ecological problems of the humus layer in the forest. — Cornell Univ. Agric. Expt Sta. Mem. 170: 1. 1935.

Tamm, C. O.: Seasonal variation in composition of birch leaves. — Physiol. Plant. 4: 461. 1951.

— Growth, yield and nutrition in carpets of a forest moss (Hylocomium splendens). — Meddelanden Statens Skogsforskn.inst. 43(1): 1. 1953.

Correction:

In vol. 9, p. 164, line 2, *instead of* increased *read* decreased.

Succinate Oxidation by Mitochondrial Preparations from Bean Seedlings

By

Alice P. Withrow and J. B. Wolff

Division of Radiation and Organisms, Smithsonian
Institution, Washington, D.C.[1, 2]
(Received March 21, 1956)

While investigating the effect of respiratory inhibitors, uncoupling agents, and other metabolic regulators on oxygen uptake and phosphorus esterification in mitochondrial preparations from bean seedlings, general observations indicated that the succinoxidase activity of the mitochondria varied considerably with the age of the seedling. Oxidative ability also appeared to vary with the organ employed to prepare the homogenate. Since the variations were so marked, it was considered desirable to investigate succinoxidase activity in relation to these factors before formulating a standard experimental procedure.

Preparation of Mitochondria

The plant homogenate was prepared by weighing out the required amount of plant material which was then placed in a cold room at $2°$ C. When cooled, the tissue was ground for approximately two minutes at $2°$ C with quartz sand and $0.4\ M$ sucrose-$0.15\ M$ sodium glycylglycine buffer (pH 7.1) (1.2 ml per gram fresh weight). The homogenate was centrifuged at $0°$ C at 500 g for 5 minutes and the supernatant liquid decanted and centrifuged at 10,000 g for 15 minutes. The resulting

Table 1. *Oxidative activity of mitochondrial preparations from 72-hour bean seedlings, with and without added inorganic phosphate.*[1]

Inorganic Phosphate	Oxygen Uptake µl/mg protein N/hr			
	Series I		Series II	
	Entire Shoot	Root	Hypocotyl Hook	Root
Added	271	480	249	467
Omitted	243	477	200	470

[1] ATP, Mg^{2+} and succinate added.

sediment was washed and re-centrifuged for 15 minutes at 10,000 g. The final pellet was suspended in 0.1 ml buffer per gram of original fresh weight and homogenized in an all-glass homogenizer. Of the final suspension, 0.5 ml was added to the side-arm of each vessel. The final concentrations of the various additions to the reaction mixture were sodium succinate, 10^{-2} M; potassium ATP, 10^{-3} M; $MgCl_2$, 10^{-3} M; KH_2PO_4, 10^{-2} M; KCl as necessary to bring the solution to required ionic concentration where any of the above were omitted. Additions of NaF, glucose, hexokinase or cytochrome c did not increase the succinoxidase activity of the seedling mitochondrial fractions from this variety of bean.

The vessels were equilibrated for 10 minutes in the water bath at 30° C and all determinations were run at this temperature for one hour. The protein nitrogen, measured according to Lowry *et al.* (2), varied somewhat according to age, but was within the range of 0.65 to 1.2 mg per ml. Inorganic orthophosphate was determined by the method of Allen (1).

Results

Comparative Oxidative Activities of Root and Shoot Mitochondria

For comparisons of the oxidative ability of root and shoot tissue, seeds of *Phaseolus vulgaris,* variety Black Valentine, were grown on glass since it was necessary to have roots free from foreign particles. The plants were germinated in the dark at 25° C and harvested after 72 hours. During the three-day germination period, the seeds were automatically subirrigated with tap water at 8-hour intervals.

Table 1 presents typical results obtained with mitochondria from root and shoot tissues of 72-hour seedlings. The ability of the mitochondria from the root to oxidize succinate was markedly greater than that of mitochondria from either the entire shoot or from the excised hyocotyl hook. Addition of phosphate did not increase the succinoxidase activity of the root mito chondria, but it increased that of the shoot mitochondria by 10 to 25 percent

Figure 1. *Succinoxidase, endogenous, and total oxidative activity of mitochondria from bean seedlings three to seven days of age.*
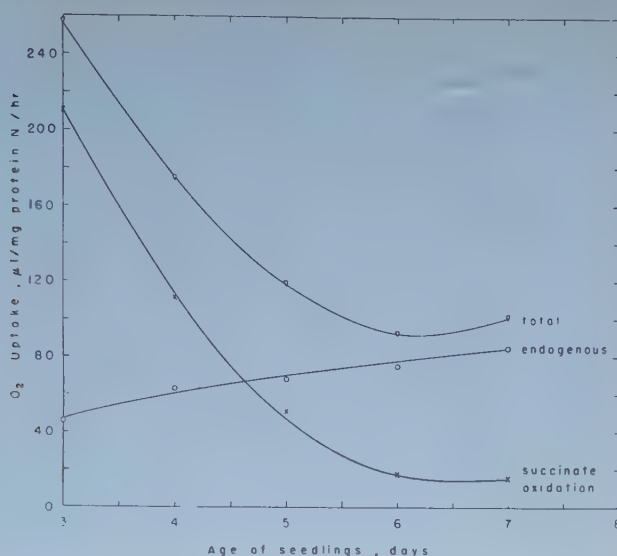
Age and Succinoxidase Activity

For a study of activity in relation to age, the excised hypocotyl hook of the bean seedling was used since its morphological structure appeared to be less altered throughout the experimental period than that of other portions of the plant. For this series, the seeds were germinated in subirrigated gravel culture on tap water in the dark at 25° C.

Figure 1 and Table 2 present data on the oxygen uptake of mitochondria from the hyocotyl hooks of bean seedlings from three to seven days after seeding, both with and without added inorganic phosphate. The endogenous oxidative activity was measured in the absence of added oxidizable substrate; the succinoxidase activity was taken to be the total oxygen uptake in the presence of succinate, less the endogenous. The figure shows the total

Table 2. *Relation of age and inorganic phosphate to the oxidative activity of mitochondria from bean hypocotyl hooks.*[1]

| Age of Seedling | Oxygen Uptake μl/mg protein N/hr | |
Days	With Inorganic P	No Inorganic P
3	257	195
4	175	124
5	119	88
6	92	80
7	101	92

[1] ATP, Mg^{2+} and succinate added.

Table 3. *Oxidative activity of mitochondria from hypocotyl hooks of 3- and 7-day seedlings in the presence and absence of ATP and Mg^{2+}.*

Age of Seedlings in Days	ATP and Mg^{2+}	Inorganic Phosphate	Oxygen Uptake μl/mg protein N/hr
3	Added	Added	205
	Omitted	Added	197
7	Added	Added	124
	Omitted	Added	128

oxygen uptake with succinate, the endogenous, and the calculated succinoxidase activities.

The mitochondria from the hooks of three-day plants had a high succinoxidase activity which decreased markedly within 24 hours; by seven days the succinoxidase activity and fallen to a low level. On the other hand, the endogenous consumption of oxygen (in the absence of succinate) was relatively low with mitochondria from three-day plants, but gradually increased until it was almost doubled by seven days and at this time accounted for the greater part of the oxygen uptake. The total oxygen uptake decreased steadily as the seedlings increased in age.

Although during this series no comparative data were taken on the effect of ATP and Mg^{2+} on oxygen consumption in the presence and absence of succinate, it was found in numerous other experiments on plants within this age limit that ATP and Mg^{2+} were not effective in increasing oxygen uptake under experimental conditions similar to those used, as shown in the data given in Table 3. Therefore, in all the reported experiments ATP and Mg^{2+} can be considered as having no effect on oxygen consumption.

Adding inorganic phosphate increased the succinoxidase activity of hook preparations from all seedlings, but the greatest increase (30 to 40 percent) occurred with mitochondria from three- to five-day plants. This was reduced to less than 10 percent with preparations from seven-day old seedlings.

No phosphate esterification was detected because the difference in inorganic phosphate concentration before and after the reaction was too small to measure with the colorimetric method employed.

Summary

1. Inorganic phosphate, in the presence of succinate, increased the oxygen uptake of mitochondrial preparations from shoots of bean seedlings, but had little effect on the oxidative activity of root preparations.

2. Inorganic phosphate significantly increased the oxidative activity of

mitochondria from shoots of very young seedlings given a succinate substrate, but its effect decreased as the age of the seedling increased.

3. Oxidative activity of root mitochondria from 72-hour bean seedlings with succinate as substrate was considerably higher than that of shoot mitochondria.

4. Succinoxidase activity progressively decreased as the seedling increased in age from three to seven days. On the other hand, the endogenous activity increased as the seedling became older, within the age limits investigated.

References

1. Allen, R. J. L.: The estimation of phosphorus. — Biochem. J. 34: 858. 1940.
2. Lowry, O. H., Rosebrough, N. J., Farr, A. & Randall, Rose: Protein measurement with Folin phenol reagent. — J. Biol. Chem. 193: 265. 1951.
3. Withrow, R. B., Withrow, Alice P. & Wolff, J. B.: An aggregate-free subirrigation method for germinating large quantities of seeds. (In press).

On the Effect of Some Protective and Stimulatory Substances in Honey-dew on the Germination of Ergot Conidia

By

A. St. Garay

Research Institute for Medicinal Plants,
Budapest XII, Dániel ut 40
(Received March 22, 1956)

On ears of rye infected by ergot sweet viscous drops, the honey-dew appear 10—14 days after infection. This honey-dew contains many ergot conidia. The problem of the origin of honey-dew has been discussed by several authors. According to some opinions it may be produced by the ergot itself (Kühn 1863), whereas others maintain the view that the honey-dew is secreted by rye tissue (Meyen 1841, Bonorden 1858). The latter view is supported also by Kirchhoff (1929), since honey-dew contains reducing sugars, according to Fuchs and Pöhm (1953) primarily invert sugars, while the presence of these substances could not be observed in sclerotia. He further states that honey-dew could never be observed under saprophytic conditions. Falck (1922) reports limited formation of honey-dew on unfertilized ears, while according to Tschermak (1906) the lack of fertilization does not cause such an effect. Literature treating the problem of honey-dew is rather limited. Apart from the important role attributed to honey-dew in the spreading of ergot, Kirchhoff (1929) pointed out that its odour derives from *iso*butyric acid, and that its osmotic presure is so high (2.33 M sugar) that germination of conidia could not take place in it. Moreover according to Kirchhoff (1929) honey-dew is slightly acid, which inhibits the formation of sclerotia. Glaz (1955) has been particularly interested in the problem of preservation of honey-dew conidia. With the use of paper chromatography Fuchs and Pöhm (1953) investigated the sugars and amino acids contained in honey-dew.

The lack of more detailed literature concerning honey-dew is astonishing because it was already known long ago, that infection of rye with honey-dew is more successfull than the use of conidia of saprophytic origin. Wiechert's (1952) high yields of ergot are at least partly due to the fact, that he infected the rye with honey-dew.

The aim of the present paper was to study the different virulence of conidia of saprophytic (*in vitro* cultures) and parasitic (honey-dew) origin. Brief communications on this questions have already been published (Garay and Kökényesy 1955, Garay 1956 a).

Materials and methods

Petkus rye was infected with an ergot strain of Hungarian origin. The honey-dew which appeared was collected. Simultaneously the ergot strain was cultured on malt-agar. Saprophytic conidia were harvested from 20—30 days old cultures by shaking the cultures with glass beads. The honey-dew was then diluted with nutrient solution (Garay 1956) in the proportion of 1 : 50 and germinated in hanging drops at 20° C. The same technique was used for parasitic, *i.e.* honey-dew, conidia. In order to study the conidia and the sap of honey-dew separately the diluted honey-dew was centrifuged, at a speed of 100 rev. per min. The supernatant liquid was poured off, the centrifuged conidia were washed with nutrient solution and centrifuged again. Conidia of saprophytic origin were treated in the same way. Thus it became possible to germinate conidia of saprophytic and parasitic origin both in pure nutrient solution and in the sap of honey-dew, in the presence of different anti-metabolites. In the use of antimetabolites the reviews of Haenel (1953) served as a basis. The following abbreviations will be used in this article: SS salicylic acid; SAAT 2-sulphanilamido-4-methylthiazole; HCH hexachlorocyclohexane; TIB triiodobenzoic acid; PAB *p*-aminobenzoic acid; PS pantothenic acid; I inositol; ET ergothioneine.

The precentage germination and the rate of germ-tube growth was determined by enumeration, resp. by mikrometer under the mikroskop. I used ten replication for each experiment, and the experiments were repeated at last three times.

Results

From the values of the controls in tables 1 and 2 it is apparent that parasitic conidia in vitro give a higher germination percentage and their germ tubes grow more rapidly than those of the saprophytic ones. The centrifuged sap of honey-dew had a stimulatory effect both on the germination of saprophytic conidia and on the growth of their germ tubes. The tables also show that SS and SAAT as well as H_2O_2 were inhibitory to these processes in the concentration ranges used; but in the presence of honey-dew

Table 1. *Per cent germination of conidia of parasitic and saprophytic origin in the presence of different antimetabolites, also stimulatory or protective effects of the sap of honey-dew. After 24 and 48 hours.*

Treatment		Conidia germinated							
		Saprophytic conidia				Parasitic conidia			
Antimeta-bolites	Conc. in M	In culture medium		In culture medium + sap of honey-dew		In culture medium		In culture medium + sap of honey-dew	
		24 h.	48 h.	24 h.	48 h.	24 h.	48 h.	24 h.	48 h.
SS	10^{-3}	0	0	2	3	93	97	93	97
SAAT	$2 \cdot 10^{-3}$	5	7	14	28	93	97	93	97
HCH	10^{-2}	0	0	0	0	20	32	19	34
TIB	10^{-2}	0	0	0	0	24	37	26	37
H_2O_2	$5 \cdot 10^{-3}$	0	0	22	52	73	90	84	96
Control		7	35	24	82	93	97	95	98

sap this inhibitory effect was less significant. The sap of honey-dew must therefore contain certain substances, which exert a protective effect against the above-mentioned antimetabolites. The inhibitory effect of HCH and TIB was not antagonised by honey-dew sap.

From these results the important question may be raised, whether the observed effect was a specific one. In order to solve this problem, it has been investigated whether the effect of antimetabolites (with the exception of TIB) might be abolished by the corresponding metabolites. As it may be seen from

Table 2. *Length of germ tubes* (microns) *of conidia of parasitic and saprophytic origin in the presence of different antimetabolites, also stimulatory or protective effects of the sap of honey-dew. After 24 and 48 hours.*

Treatment		Conidia germinated							
		Saprophytic conidia				Parasitic conidia			
Antimeta-bolites	Conc. in M	In culture medium		In culture medium + sap of honey-dew		In culture medium		In culture medium + sap of honey-dew	
		24 h.	48 h.	24 h.	48 h.	24 h.	48 h.	24 h.	48 h.
SS	10^{-3}	0	0	105	125	225	350	250	380
SAAT	$2 \cdot 10^{-3}$	28	32	160	185	163	630	225	590
HCH	10^{-2}	0	0	0	0	105	165	105	165
TIB	10^{-2}	0	0	0	0	130	230	180	240
H_2O_2	$5 \cdot 10^{-3}$	0	0	325	480	235	650	420	700
Control		105	350	320	430	240	700	420	700

Table 3. *Effect of antimetabolites and their antagonists on germination of ergot conidia of saprophytic origin and the length of the germ tubes.*

Treatment		Conc. in M	After 15 hours		After 40 hours	
			%	microns	%	microns
Antimetabolites	SS	10^{-3}	0	0	0	0
	SAAT	$2 \cdot 10^{-3}$	5	28	60	80
	HCH	$5 \cdot 10^{-3}$	3	14	51	400
	H_2O_2	$5 \cdot 10^{-3}$	0	0	2	75
Metabolites	PAB	10^{-3}	13	41	76	560
	PS	10^{-3}	14	45	82	590
	I	$5 \cdot 10^{-3}$	13	45	82	590
	ET	$5 \cdot 10^{-3}$	15	45	93	650
Antimetabolites + metabolites	SS + PS	$\left\{ \begin{matrix} 10^{-3} \\ 10^{-3} \end{matrix} \right\}$	2	12	4	80
	SAAT + PAB	$\left\{ \begin{matrix} 2 \cdot 10^{-3} \\ 10^{-3} \end{matrix} \right\}$	5	41	75	590
	HCH + I	$\left\{ \begin{matrix} 5 \cdot 10^{-3} \\ 5 \cdot 10^{-3} \end{matrix} \right\}$	4	17	53	390
	H_2O_2 + ET	$\left\{ \begin{matrix} 5 \cdot 10^{-3} \\ 5 \cdot 10^{-3} \end{matrix} \right\}$	3	39	21	560
Control		—	16	46	81	550

table 3 the metabolite did not stimulate germination, but on the other hand, the effects of antimetabolites, except that of the HCH, were, at least partially, eliminated by the corresponding compounds. It is of considerable interest to note that the effect of H_2O_2 in inhibiting germination was only partially compensated by ET, but growth inhibition was entirely abolished.

Discussion

The main question to be discussed is, what explanation could be given for the protective and stimulating effect of honey-dew. Numerous vitamins having been detected in rye (Carrol and Peng 1951), it may be assumed that honey-dew also contains them. Special attention has been given to the problem of processes leading to the abolition of the toxic effect of hydrogen peroxide.

As shown from the data presented earlier the activity of catalase and peroxidase in rye is profoundly altered in infected plants (Garay 1955 b). This circumstance suggests, — as assumed also by Rubin and Arzichowskaja (1953) — that the above-mentioned enzymes as well as the peroxide metabolism are playing a definite role in the struggle against infection.

Table 4. *Catalase activity and ergothioneine contents of conidia of saprophytic and parasitic origin.*

Material used		mg. H_2O_2 decomposed	Ergothi- oneine %
Honey-dew	Centrifuged sap	4.54	0.045
	Conidia	0.27	traces
Saprophytic culture	Conidia	0.30	traces

This view is supported by the data described, inasmuch as it has been found that ergot conidia do not germinate in the presence of H_2O_2. The protective nature of ergothioneine was proved originally by Mann and Leone (1953) in the course of chemical investigations on bull semen. It seemed to be very probable, that the protective nature of ergothioneine, which compound is characteristic of ergot, may exert its effect in the course of infection. Up to the present the presence of ergothioneine in honey-dew had not been demonstrated, therefore this task had to be performed. Table IV indicates the ergothioneine content of honey-dew and saprophytic conidia and the values of catalase activity. I determined ergothioneine content by Hunters (1949) diazo method, and the activity of catalase by that of Belosersky and Proskuryakow (1951). The latter assay was carried out, because it had been observed that honey-dew sap added to hydrogen peroxide caused a strong evolution of gas. From the table it is evident that honey-dew contains a significant amount of ergothioneine, and that the catalase activity of honey-dew sap is also high, whereas the same experiments carried out with washed conidia and conidia of saprophytic origin gave low values. It must be noted, that the diazo reaction is disturbed by unknown substances in honey-dew. It is extremely striking that, while the colour obtained with pure ergothioneine shows a single absorption maximum (filter S 53 of a Pulfrich photometer), the colour obtained with honey-dew gives two maxima (with filters S 53 and S 43).

Summary

1. Conidia of honey-dew in vitro germinate better than conidia of saprophytic origin.

2. The sap of honey-dew stimulates germination, and contains substances protective against salicylic acid, sulphomethylthiazole, and H_2O_2. As protective compounds ergothioneine and catalase could be identified. These substances antagonize the inhibitory effect of H_2O_2.

References

Belosersky, A. & Proskurjakov, N.: Praktitischeskoie rukowodstwo po biochimyi rastenii. — Sow. Nauka. Moskwa. 1951.

Bonorden, H.: Beobachtungen über die Bildung von Spermodia clavus (Secale cornutum). — Bot. Ztg. 16: 97. 1858.

Carrol, J. & Peng, L.: Comparation of the niacin and panthothenic acid content of certain cereal grains. — Science 113: 211. 1951.

Falck, R.: Über die Bekämpfung und die Kultur des Mutterkorns im Roggenfelde. — Pharm. Ztg. 67: 777. 1922.

Fuchs, M. & Pöhm, M.: Zur Kenntniss des Honigtaus. — Scientia Pharm. 21: 239. 1953.

Garay, A.: Untersuchungen über die Beziehungen zwischen Mutterkorn und Roggen. — Naturwiss. 42: 422. 1955.

— Role of ergothioneine and catalase in infection by ergot fungus. — Nature. 177: 91. 1956 a.

— Studies on the effect of ergot infection on rye and on toxic substances in the sclerotium. — Phytopath. Z. (in press).

— & Kökényesv, S.: Vergleichende Untersuchungen über die Keimung der Mutterkornkonidien saprophytischen und parasitischen Ursprungs.-Phytopath. Z. 25: 109. 1955.

Glaz, E.: Researches about the viability and preservation of ergot conidia of Claviceps purpurea grown in submerged culture. — Acta Mikrobiol. Hungarica. 2: 315. 1955.

Haenel, H.: Antiwirkstoffe I—II. — Die Pharmazie. 8: 891 and 991. 1953.

Hunter, G.: The determination of ergothioneine in simple solution and in blood. — Canad. J. of Res. E. 27: 230. 1949.

Kirchhoff, H.: Beiträge zur Biologie und Physiologie des Mutterkorns. — Zbl. Bakteriol. und Parasitenkunde. II. 77: 310. 1929.

Kühn, J.: Untersuchungen über die Entwicklung, das künstlichen Hervorrufen und die Verhütung des Mutterkorns. Mitt. a. d. Physiol. Lab. u. d. Versuchstation d. Landw. Inst. Univ. Halle. 1863.

Mann, T. & Leone, E.: Studies on the metabolism of semen. — Biochem. J. 53: 140. 1953.

Meyen, N.: Pflanzenpathologie. — Berlin. 1863.

Rubin, B. & Arzichowskaja, E.: Biochemische Charakteristik der Wiederstandsfähigkeit der Pflanzen gegenüber Mikroorganismen. — Akad. Verl. Berlin. 1953.

Tschermak, E.: Die blüh-, und Fruchtbarkeitverhältnisse bei Roggen und Gerste, und das Auftreten von Mutterkorn. — Fühlings. Landw. Ztg. 55: 194. 1906.

Wiechert, E.: Mutterkornanbau in Meklenburg. — Die Pharmazie. 7: 859. 1952.

The Germination of Ergot Conidia as Affected by Host Plant, and the Culture of Ergot on Excised Roots and Embryos of Rye

By

A. St. Garay

Research Institute for Medicinal Plants,
Budapest XII, Dániel ut 40
(Received March 22, 1956)

The relation between rye and ergot may be investigated from two different point of views: 1) What is the effect exerted by infection on host plants? 2) In what is shown the effect of the host plant on the life of parasites? I have already given an account of investigations concerning the first point (Garay 1955). The present discussion deals with the second question. Earlier experiments on the relation between rye and ergot were intended to determine the stage of development at which rye is most susceptible to infection by ergot (Engelke 1902). Kirchhoff (1929) in particular treated this question in detail, and stated that the infection by ergot is generally more successful in the first stage of flowering than later. Bonns (1922) endeavoured to culture ergot on rye kernels in vitro. Similar experiments have been reported by Kreitmair and Kussner (1931) and by Schweizer (1941). On the basis of their work Michener and Snell (1950) added to saprophytic cultures of ergot an extract of rye seddlings, filtered through a Seitz filter, and stated that in this case the growth of fungus was accelerated. The growth stimulating effect of rye homogenate was investigated first of all by Berman and Youngken (1954). According to them an ear extract generally stimulates the growth of saprophytic cultures of ergot. The extract from mature ears had a more marked effect, than that of ears in the budding or flowering phases, which is somewhat contrary to the above mentioned data of Kirchhoff. The

active substance may be extracted by acetone and ether. Partly on the basis of these investigations I started my experiments.

Materials and methods

Test plants: Petkus rye and Bánkuti 1002 wheat; ergot race number 184. Ergot cultures were prepared in the usual way, and were allowed to grow on malt agar culture medium. For both infection and germination experiments (in hanging drops) conidia from 20—25 day old cultures shaken with sterile glass-beads were used. Germination took place at 20° C on the culture medium shown below:

KH_2PO_4	1.0 g	Asparagine	2.0 g
$MgSO_4 \cdot 7H_2O$	0.6 g	Saccharose	30.0 g
$CaCl_2$	0.5 g	Dist. water	1000.0 ml

The culture medium was adjusted to different pH values with citric acid and sodiumphosphate in order to determine the pH optimum for germination of conidia. The pH of tissues slices was determined by different indicators, the osmotic pressure was determined by plasmolysis methods. In view of the extraordinary variability of ergot I used ten replicates for each experiment, and the experiments were repeated at least three times.

Results

The pH. Figure 1 shows the pH dependence of germination of ergot conidia and the length in microns of hyphae developed during 48 hours.

As can be seen from the graphs, the maximal values are attained at pH 4.8 both in percentage of germinated conidia and in the length of germ tubes.
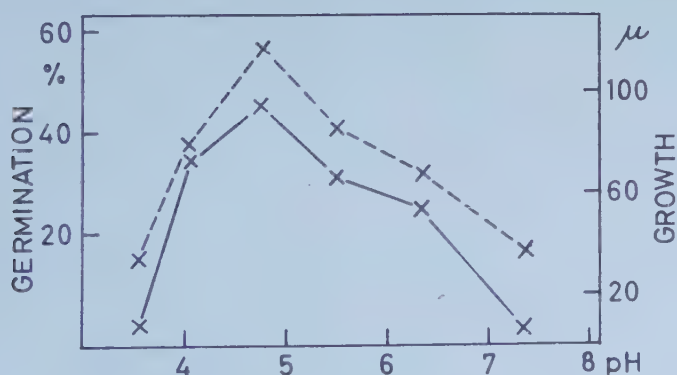

Figure 1. *Effect of pH on the germination* (————) *and the growth of germ tubes* (- - - - -) *of ergot conidia.*

Table 1. *The pH and osmotic pressure of different organs of rye.*

Material		pH	Osmotic pressure
Seedling	coleoptile	5.2—5.7	9.07
	root	5.2—5.7	9.07
Fully developed plant	node	5.2—5.7	3.35
	internode	4.0—5.0	9.28
	style	5.4—6.2	—
	glume	4.0—5.0	—

This value is pH 0.4—0.6 lower then estimated by others. The explanation for this may be sought, in my opinion, in the extraordinary variability of ergot and, on the other hand, in the circumstance that only the pH optimum for the growth of saprophytic cultures, and not that for germination, was determined by the authors mentioned. Growth and germination are not dependent on pH in quite the same way. If we examine the values of the graphs from this viewpoint, then we are able to state that only 1.5 per cent of conidia germinate at pH 3.3, *i.e.* only 3 per cent of the optimal value. At the same pH the length of germ tubes is 38 microns, *i.e.* 32 per cent of the optimal value. At other pH levels the same comparison held. This means therefore that curve showing the relationship between germination and pH is much steeper, then that for the growth of germ hyphae.

Table 1 shows the pH of different tissues of rye. As may be seen, all values fall into a range in which ergot conidia germinate well, although only pistils. stamens, lodiculae (Békésy 1939) internodes and nodes are susceptible to infection. Stoll and Brack (1944) give an account of the susceptibility of nodes, while I performed experiments showing the susceptibility of inter-nodes. Figure 2 shows sclerotia growing on nodes and internodes.

Osmotic pressure. The optimal osmotic pressure for germination of conidia was determined on pure cane sugar solutions. The results are presented in Figure 3.

The second column of table 1 shows the osmotic pressure of different tissue sections of rye. That of the node, as may be seen, approaches the optimum for germination of conidia.

Effect of rye extract on germination of ergot conidia and on the growth of saprophytic cultures of ergot. In a series of experiments it has been proven that the extract prepared from detached parts of rye [fresh matter] with dist. water in the proportion of 1 : 25 does not stimulate the germination of ergot conidia, even if sterilized by autoclaving or filtration. This contradict the above mentioned data (Berman and Youngken 1954) showing that the extract stimulates the growth of saprophytic cultures. Results of my own

Figure 2. *Sclerotia grow-ing on internodes and nodes of rye.*
[Photo P. Szalay]

experiments carried out on this question are shown in table 2. In this case saprophytic cultures were cultured on 100 ml, diluted malt-extract in Kolle flasks. To the experimental cultures 2 g powdered rye (dry matter) was added. The media were sterilized in an autoclave (1 atm., 20 min).

As may be seen from the table the growth of ergot is initially stimulated by the extract of ears, leaves, stems, nodes and roots. The strongest effect was obtained in the case of ears. On cultures older then 20 days, it was obviously not possible to observe stimulating effect. It is noteworthy that neither in the treated nor in the control cultures could any alkaloid be found by the van Urk reaction.

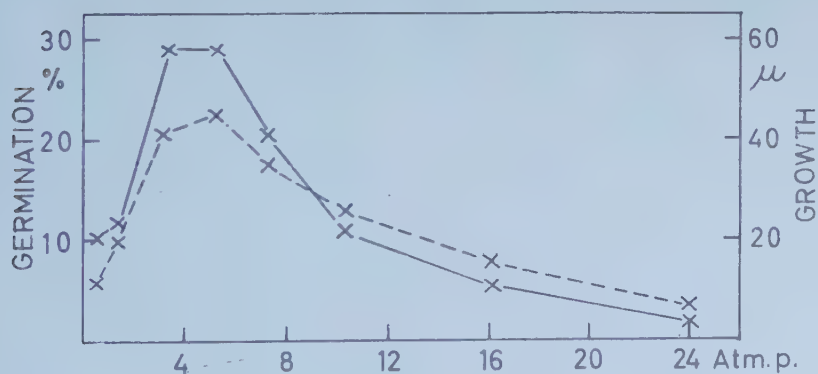There emerges the question, whether infection produces in rye a substance

Figure 3. *Effect of the osmotic pressure on the germination (———) and the growth of germ tubes (- - - - -) of ergot conidia.*

Table 2. *The effect of rye extracts on the growth of saprophytic cultures of ergot.*

Organs	g. dry matter			
	after 10 days	after 20 days	after 40 days	after 60 days
Control	0.361	1.082	1.383	2.109
Ear	0.723	1.122	1.420	2.141
Leaf	0.585	1.057.	1.281	2.250
Internode	0.572	1.088	1.265	2.118
Node	0.576	1.160	1.360	2.282
Root	0.560	1.087	1.350	2.202

which is inhibitory to the growth of ergot. To decide this problem the malt culture medium was enriched with the extract of infected and healthy rye. On both extracts the ergot developed at a similar rate.

It is of interest to note, that although the growth of saprophytic cultures is stimulated by the extracts mentioned, no higher yield of ergot could be obtained when the conidia before inoculation of rye were suspended in malt+ear extract in comparison with the control (malt). Using the above mentioned suspensions, 36 m² plots of rye were infected in five replications and in every case the ergot yield remained the same within the limits of error. The infections were carried out according Békésy's method (Békésy 1938).

Growth of saprophytic cultures of ergot on excised roots of wheat and on embryos of rye. Rye embryos have been cultured in the usual way by the method of Gautheret (1935). The cultivation of isolated roots of rye being very difficult, wheat roots were cultured by Gautheret's (1935) methods. Attempts were made to infect isolated roots and embryos. Ergot grows equally well in the presence of roots and embryos; its hyphae grow around the roots and embryos without penetrating into them, even when roots and embryos were injured with a needle. It was therefore impossible to infect isolated roots and embryos with ergot. In such »complex cultures» no kind of micro-sclerotia (Engelke 1902) or sclerotia formation could be observed. The cultures did not contain any alkaloid detectable by the Urk reaction.

It may be stressed that the growth of roots was inhibited when the inoculated ergot culture developed to a certain degree. The cause of this is not, as I have already indicated (Garay 1956), that the saprophytic cultures of ergot contain substances inhibitory to the growth of roots, but as we may assume, that the culture formes a complete impermeable layer excluding oxygen from the roots. So far this assumption has not been proved experimentally.

Summary

1. The optimal pH value for the germination of ergot conidia is 4.8 in the presence of citric acid sodiumphosphate buffer. The growth of germ tubes is not as sensitive to pH as the germination percentage.

2. Ergot is not only able to live as a parasite on ears and nodes but also on internodes.

3. Germination of ergot conidia proceeds most satisfactorily at an osmotic pressure of 3.8 atm.

4. Extracts from different parts of the rye plant did not stimulate germination of ergot conidia.

5. Autoclaved extracts of different organs of the rye plant stimulated the growth of saprophytic cultures of ergot. The strongest effect was shown by extracts of ears.

6. Extracts of infected as well as of healthy rye stimulated the growth of saprophytic cultures of ergot. It was thus impossible to show by this method that in ergot as a result of infection substances injurious to the parasite might be produced.

7. Saphophytic cultures grew well in cultures of rye embryos and on isolated wheat roots, but did not affect the host-plant. In such cultures it was not possible to detect the presence of alkaloids.

References

Békésy, M.: Über parasitische Mutterkornversuche. — Zbl. Bakteriol. u. Parasitenkunde II. 99: 321. 1938.
— Untersuchungen über den Alkaloidgehalt des Mutterkorns. — Biochem. Z. 302: 187. 1939.
Berman, M. & Joungken, H.: The use of certain rye homogenates for supporting growth of Claviceps purpurea Tul. — J. Amer. Pharm. Assoc. 43: 200. 1954.
Bonns, W.: Preliminary study of Claviceps purpurea in culture. — Am. J. Bot. 9: 339. 1922.
Engelke, E.: Neue Beobachtungen über Vegetationsformen des Mutterkornpilzes Claviceps purpurea. Beiblatt z. Hedwigia 51: 221. 1902.
Garay, A. St.: Untersuchungen über die Beziehungen zwischen Mutterkorn und Rogen. — Naturwiss. 42: 422. 1955.
— Studies on the effect of ergot infection rye and on toxic substances in the Sclerotium. — Phytopath. Z. 1956. (In press.)
Gautheret, R.: Rechérches sur la culture des tissus végétaux. — These, Univ. Paris. 1935.
Kirchhoff, H.: Beiträge zur Biologie und Physiologie des Mutterkornpilzes. - - Zbl. Bakteriol. und Parasitenkunde II. 77: 310. 1929.
Kreitmair, H. & Kussner, W.: Über den Alkaloidgehalt von Claviceps purpurea bei Kultivierung auf künstlichen Nährboden. — Biochem. Z. 129: 189. 1931.
Michener, H. & Snell, N.: Studies on cultural requirements of Claviceps purpurea and inactivation of ergotamin. — Am. J. Bot. 37: 52. 1950.
Schweizer, G.: Über die Kultur von Claviceps purpurea. — Phytopath. Z. 4: 137. 1941.
Stoll, A. & Brack, A.: Zum feldmässigen Anbau von Mutterkorn. — Pharm. Acta Helvetica 19: 118. 1944.

Effects of Irradiation, Maleic Hydrazide, Temperature, and Age on Enzyme Activity in Seedlings of Corn (*Zea mays* L.)

By

Francis A. Haskins and Harold W. Chapman

Departments of Agronomy and Horticulture,
University of Nebraska, Lincoln, Nebraska
(Received March 22, 1956)

The work of Schmidt and Frolik (1951) and of Beard (1955) has shown that plants of corn (*Zea mays* L.) grown from seeds treated with appropriate doses of X-rays or thermal neutrons are greatly reduced in stature and survival in comparison with control plants, and that pronounced increases in chromosomal abnormalities and pollen sterility also result from seed irradiation. It is reasonable to suspect that these morphological and cytological effects might be associated with changes in the metabolism of the plant. Accordingly, a series of experiments has been conducted in which preparations of normal corn seedlings and preparations of dwarfed seedlings grown from irradiated seeds have been compared with respect to several enzyme activities. Measurements have also been made using seedlings which were stunted by the effects of seed treatment with maleic hydrazide, and other seedlings which were stunted by subjection to lowered temperature during growth.

The enzyme activities which have been studied are catalase, cytochrome oxidase, peroxidase, acid phosphatase, and polyphenolase. The influence of different types of radiation on various enzyme activities, including some of those listed above, in certain mammals and microorganisms has been the object of rather extensive studies (*e.g.* Patt, 1954, and Powell and Pollard, 1955). Obviously, however, irradiating a dormant seed, planting the seed,

and using the resulting seedling for enzyme assays is quite different from irradiating a rat or mouse, removing certain tissues or organs, and using these in enzyme studies, or irradiating a population of bacterial cells and immediately using these cells in making an enzyme preparation. Although Mikaelsen and Halvorsen (1953) observed a decrease in the rate of respiration of germinating barley seeds resulting from treatment of the dormant seeds with X-rays, their report does not include information regarding specific enzyme activities. There is very little information available concerning the influence of seed treatment on the enzymatic properties of seedlings grown from the treated seeds.

Materials and Methods

The corn hybrid, L289×I205, was used in these investigations. Seedlings were grown in vermiculite which was saturated with nutrient solution (solution 1 of Hoagland and Arnon, 1950, supplemented with trace minerals) immediately after planting and was kept moist with tap water for the remainder of the growing period. All seedlings except those in the temperature study were grown in the laboratory at room temperature, which varied between 22° and 35° C during the course of these experiments, under Ken-rad Cool White Standard fluorescent tubes. Light intensity at the level of the seedlings was approximately 1000 foot candles. A 14-hour photoperiod was used.

Seedlings to be used in enzyme preparations were excised at the surface of the vermiculite and the remains of the coleoptiles adhering to the stems were removed and discarded. The term green seedling, as used in this report, therefore refers to only that portion of the seedling above the coleoptilar node.

Seed irradiation. Seeds were sent to the Brookhaven National Laboratory for irradiation. We are indebted to Dr. Seymour Shapiro for conducting the irradiations. For the X-ray treatments, seeds were exposed in a single layer 27 cm from the X-ray source. The X-ray facility was operated at 250 KVP and 30 ma, and the X-rays were filtered through 1 mm of aluminum. The intensity of irradiation at the surface of the seeds was approximately 900 r/min.

Thermal neutron treatments were administered in the thermal column of the nuclear reactor at Brookhaven. Dosage determinations were based on the radioactivity induced in gold foils included with the seeds during treatment. The flux was such that an exposure of 12 hours was required for the highest neutron dosage. The cadmium ratio in the thermal column was approximately 5000 : 1 and the gamma ray contamination approximately 50 r/hour.

Seeds were packaged in moisture-proof containers for mailing to and from Brookhaven, and following their return the seeds were stored in a desiccator over $CaCl_2$ until they were planted.

Maleic hydrazide treatments. Seeds were soaked at room temperature for 24 hours in solutions of the diethanolamine salt of maleic hydrazide (MH 30, U.S. Rubber Company) of various concentrations, then rinsed thoroughly with distilled water and planted in vermiculite.

Temperature treatments. Seedlings were started at room temperature and after

attaining a height of approximately 60 mm, they were moved to illuminated constant temperature chambers. Temperature control in these chambers was within limits of $\pm 0.5°$ C. Fluorescent tubes were used for illumination. The light intensity was similar to that which was used for the other seedlings, and a 14-hour photoperiod was used.

Enzyme assays. The methods which were used in making enzyme preparations and assaying for activities of catalase, cytochrome oxidase, peroxidase, phosphatase, and polyphenolase as well as content of protein nitrogen have been described earlier (Haskins, 1955). All determinations were made in duplicate. Differences between duplicate determinations seldom exceeded 5 per cent in peroxidase, polyphenolase, and phosphatase assays. In cytochrome oxidase and catalase assays, however, duplicate determinations occasionally differed by as much as 20 per cent, particularly when preparations of very low activity were involved.

Results and Discussion

The data presented in table 1 show that seed treatment with X-rays, thermal neutrons, or maleic hydrazide resulted in a reduction of seedling stature. Similarly, small seedlings were obtained from non-treated seeds by lowering the temperature during growth or by shortening the growth period. In appearance, however, seedlings from treated seeds had extensive leaf mottling and were generally much less vigorous than seedlings which were small because of lowered temperature or reduced growing period. The latter seedlings were essentially free of mottling. Metabolic differences among the small seedlings are indicated by the fact that the seedlings most drastically reduced in stature as a result of seed irradiation or maleic hydrazide treatment yielded preparations appreciably lower in protein nitrogen than preparations of seedlings of comparable stature from the age and temperature series.

Moreover, the seedlings which were small because of the effects of age or temperature differed enzymatically from those which were small by virtue of the effects of seed treatment with X-rays, thermal neutrons, or maleic hydrazide. The height reductions which resulted from the irradiation and maleic hydrazide treatments were found to be associated with appreciable increases in the specific activities of peroxidase, acid phosphatase, and polyphenolase; while the height reductions resulting from the age and temperature treatments were usually accompanied by slight decreases in these three types of enzyme activity (figures 1, 2, 3). With respect to catalase and cytochrome oxidase, on the other hand, increased enzyme activity and reduced seedling height appeared to be associated for all treatments (figures 4, 5).

Although seed treatment with X-rays, thermal neutrons, or maleic hydra-

Table 1. *Ages, heights, and fresh weights of green seedlings of L289×I205 corn, and protein nitrogen contents of enzyme preparations from these seedlings.*

Source of material	Age (days)	Seedling height (mm)	Fresh weight (gm)	Protein N per ml preparation (mg)
Age series	11	265	1.15	0.23
	9	213	0.90	0.26
	7	131	0.57	0.30
	5	39	0.08	0.38
Temperature series				
30° C	9	295	1.32	0.19
24	9	214	0.86	0.22
18	9	125	0.54	0.28
12	9	93	0.42	0.30
X-ray series				
Control	9	266	1.58	0.23
10,000 r	9	256	1.26	0.24
20,000 r (a)	9	181	0.93	0.24
20,000 r (b)	9	91	0.38	0.23
Thermal neutron series				
Control	8	270	1.09	0.24
1.2×10^{13} N_{th}/cm^2	8	204	0.84	0.27
2.2×10^{13} »	8	113	0.44	0.29
3.8×10^{13} »	8	56	0.22	0.30
Maleic hydrazide series				
Control	8	271	1.29	0.23
1.5×10^{-3} M solution	8	171	0.72	0.18
3.0×10^{-3} » »	8	74	0.23	0.21
6.0×10^{-3} » »	8	38	0.07	0.19

zide resulted in altered enzymatic activities in the green seedlings, it is improbable that these treatments exerted a direct influence on the enzymes studied in these experiments. This conclusion is based on measurements (unpublished results from this laboratory) of the five types of enzyme activity in preparations of embryos from control, X-ray-treated, and thermal neutron-treated seeds, and of etiolated shoots from control seeds and seeds treated with X-rays, thermal neutrons, or maleic hydrazide. These assays, in contrast to those employing green seedlings, failed to show large or consistent differences with respect to any of the enzyme activities studied. Thus it appears that the irradiation and maleic hydrazide treatments of corn seeds bring about primary changes of an unknown nature, and that among the manifestations of these unknown changes are altered patterns of polyphenolase, peroxidase, and phosphatase activity in the green seedling. Although the data indicate certain similarities in the effects of X-rays, thermal neutrons, and maleic hydrazide, the primary metabolic changes induced by the three agents may, of course, be quite dissimilar.
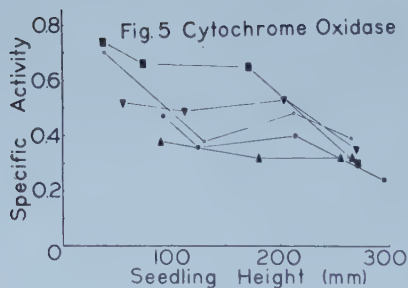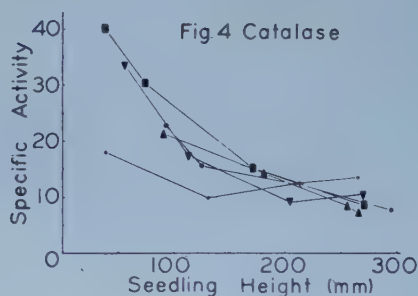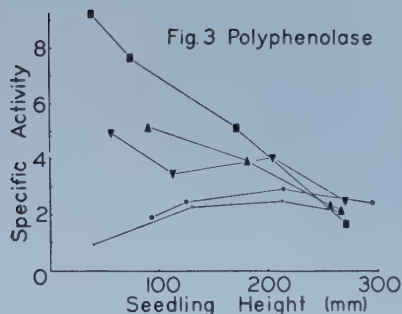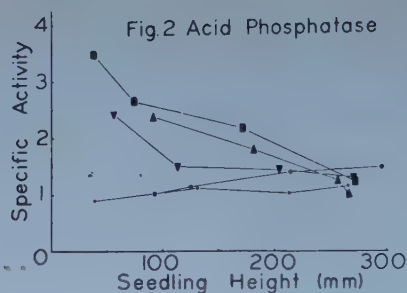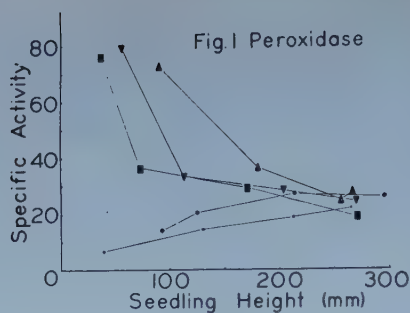
Figures 1—5. *Relationships between seedling height and five types of enzyme activity for corn seedlings which varied in height because of the effects of age* O———O, *temperature* ● ———●, *X-rays* ▲ ———▲, *thermal neutrons* ▼———▼, *and maleic hydrazide* ■———■. Specific activities are expressed in the following units per minute per mg protein N: peroxidase — increase in optical density at 460 mμ; acid phosphatase — micromoles *p*-nitrophenol liberated; polyphenolase — increase in optical density at 410 mμ; catalase — micromoles H_2O_2 destroyed; cytochrome oxidase — micromoles cytochrome *c* oxidized.

MacKey (1951) and Caldecott, Frolik, and Morris (1952) have reported that in barley grown from X-ray- and neutron-treated seeds, the frequency of chromosomal abnormalities in relation to reductions in plant height or survival is distinctly less for X-ray treatments than for neutron treatments. On the basis of this observation they have suggested that X-rays bring about pronounced physiological or extra-chromosomal effects which are not found following neutron irradiation. Beard (1955) has reported similar data for corn. Since the present experiments have not demonstrated any large differences between the two types of radiation, the suggested physiological effect is apparently not closely related to any of the enzyme activities considered here.

Summary

Corn seedlings of various heights were obtained from seeds treated with various dosages of X-rays, thermal neutrons, or maleic hydrazide, and also from non-treated seeds by adjusting duration of the growth period or temperature during growth. These seedlings were used in making enzyme preparations, and the activities of the preparations with respect to catalase, cytochrome oxidase, peroxidase, acid phosphatase, and polyphenolase were determined. It was found that seedlings which were small as a result of seed treatment with X-rays, thermal neutrons, or maleic hydrazide yielded enzyme preparations which were appreciably higher than controls in peroxidase, phosphatase, and polyphenolase activities, while seedlings which were small because of age or temperature effects yielded preparations which were relatively low in these three enzyme activities. Assays of catalase and cytochrome oxidase, on the other hand, disclosed that with reduced seedling height there was a tendency toward increased activity regardless of the treatment employed to effect the reduction in seedling height.

References

Beard, B. H.: A comparison of the effects of X-rays and thermal neutrons on the dormant seeds of barley, maize, mustard, and safflower. — Ph. D. Dissertation, University of Nebraska. 1955.

Caldecott, R. S., Frolik, E. F. & Morris, R.: A comparison of the effects of X-rays and thermal neutrons on the dormant seeds of barley. — Proc. Nat. Acad. Sci. 38: 804. 1952.

Haskins, F. A.: Changes in the activities of several enzymes during germination and
    seedling development in corn (Zea mays L.). — Plant Physiol. 30: 74. 1955.
Hoagland, D. R. & Arnon, D. I.: The water-culture method for growing plants without
    soil. — California Agricultural Experiment Station Circular 347 Revised. 1950.
MacKey, J.: Neutron and X-ray experiments in barley. — Hereditas 37: 421. 1951.
Mikaelsen, K. & Halvorsen, H.: Experiments on the respiration of X-irradiated barley
    seeds. — Physiol. Plant. 6: 873. 1953.
Patt, H. M.: Radiation effects on mammalian systems. — Ann. Rev. Physiol. 16: 51. 1954.
Powell, W. F. & Pollard, E.: Radiation inactivation of enzymes inside and outside intact
    cells. — Radiation Research 2: 109. 1955.
Schmidt, J. W. & Frolik, E. F.: The effects of thermal-neutron irradiation of maize and
    barley kernels. — University of Nebraska Research Bulletin 167. 1951.

The Spontaneous Inactivation of Pea Root Peroxidase and its Acceleration by Coenzyme A[1]

By

Helen H. Sharpensteen, A. W. Galston, and S. M. Siegel[2]

Josiah Willard Gibbs Research Laboratories
Plant Science Department
Yale University
New Haven, Connecticut
(Received April 3, 1956)

Introduction

Peroxidase is frequently described as an unusually stable enzyme (8), being remarkably resistant to agents like heat, which usually produce complete inactivation of enzymatic activity. However, in the course of experiments on the induction of peroxidase activity in pea roots by the plant growth hormone, indoleacetic acid (IAA) (2), we noted that the peroxidase activity of the *brei* declined rapidly. The inclusion of *p*-chloromercuribenzoic acid (PCMB) in the grinding medium prevented this, implying that some sulfhydryl compound present in the *brei* was involved in the inactivation. A survey of the more common sulfhydryl compounds revealed coenzyme A to be a potent inactivator of the peroxidase of pea root *brei* and of several other enzymes. The present paper describes what is known of the nature and significance of this inactivation.

---

[2] Present address: Biology Dept., University of Rochester, Rochester, New York.

Materials and Methods

Roots of 3—4-day-old pea seedlings (var. Alaska) grown in the dark in vermiculite at 28° C. were washed in tap water and the terminal five millimeters were excised. All harvesting and excising of roots was done under weak green light which was inactive both phototropically and photomorphogenically. The root tips were ground in a mortar with washed sea sand in ice-cold 0.1 M KH_2PO_4, and the resulting *brei* was centrifuged for ten minutes at *ca.* 3000 xg. The supernatant fluid was used as the peroxidase preparation.

For measurement of peroxidase activity, 0.1—0.5 ml. of the enzyme solution was pipetted into a Klett colorimeter tube containing one ml. H_2O_2 (final concentration, 0.018 M) and sufficient buffer (0.01 M phosphate, pH 4.5) to bring the volume to 9.9 ml. At zero time 0.1 ml. of 0.5 M pyrogallol was pipetted into the tube, which was rapidly inverted to insure thorough mixing. The formation of the yellow oxidation product, purpurogallin, was followed at 15 second intervals on the Klett colorimeter equipped with a No. 42 blue filter. Enzyme activity was expressed in terms of the amount of color developed in 30 seconds; over this period of time, rate of color development is linear.

For measurement of catalase activity, 0.1 ml. of crystalline beef liver catalase (Worthington Biochemical Co.) was pipetted at zero time into ten ml. of H_2O_2-buffer mixture (0.01 M phosphate, pH 6.95, 0.009 M H_2O_2). At 30-second intervals two-ml. aliquots of the reaction mixture were pipetted into flasks containing 0.5 ml. of 5 N sulfuric acid. Residual H_2O_2 was determined by titration with 0.01 M permanganate. The activity of the enzyme was expressed by the k value, which is calculated from the equation, $k = 1/t (\log a_0/a_x)$ for the monomolecular reaction (1).

In experiments to determine temperature characteristics of the inactivation of catalase by coenzyme A (Pabst product), both the incubation of the enzyme with coenzyme A and the measurement of its activity were carried out at the desired temperature. For the 30° C. experiment, an Aminco Dubnoff metabolic shaking incubator was used, for the 20° C. and 10° C. experiments, controlled temperature rooms were used, and for the 0° C. experiment the incubation and reaction mixtures were kept in ice water baths.

Spectrophotometric determinations were made with the Beckman model DU spectrophotometer, catalase concentration being calculated from the optical density reading at 405 mm using $E\frac{405}{mM} = 78.5$ (4).

Results

1. Inactivation of peroxidase in pea root *brei*.

The time course of inactivation of peroxidase is shown in Figure 1. At a temperature of 30° C., there is a spontaneous decline in activity of the peroxidase such that its half-life is about 35 minutes. In the presence of 10^{-4} M CoA the half-life is shortened to about seven minutes, while with 10^{-3} M CoA it is about three minutes. Measurements made over a period of six hours indicate that the activity of preparations approaches the same final level in the presence or absence of CoA.
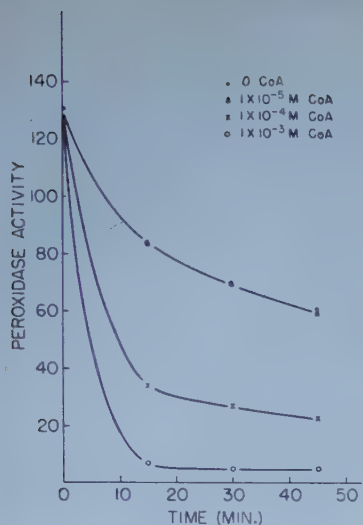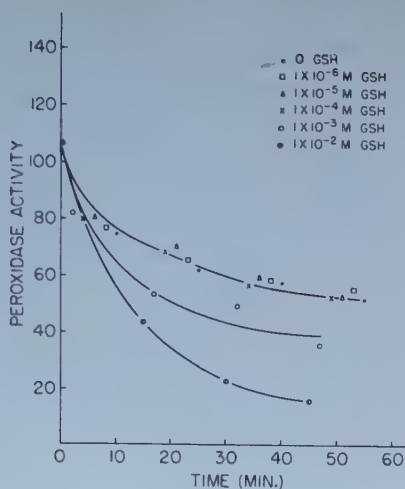
Figure 1.

Figure 2.

Figure 1. *The effect of coenzyme A on the peroxidase activity of pea root brei.*
Ordinate: Klett units.
Abscissa: Time of incubation of enzyme with inhibitor. Activity is expressed in terms of
    color formed in 30 sec. after addition of pyrogallol.

Figure 2. *The effect of glutathione on the peroxidase activity of pea root brei.* Cf. Figure 1.

The effect of CoA on peroxidase activity was compared with that of several
other compounds containing the sulfhydryl group. As Table 1 indicates,
cysteine was ineffective at the concentration levels tested while glutathione
was effective only at concentrations of 10^{-3} M or higher. Figure 2 shows the
results of a separate experiment utilizing a wider concentration range of
glutathione. It is clear that while glutathione does increase the rate of
spontaneous inactivation of peroxidase, concentrations much larger than
those of CoA must be used to be equally effective. In still another experiment,
10^{-4} M 2,3-dimercaptopropanol (BAL), was found to be without effect on
the enzyme.

The inhibitory action of coenzyme A on peroxidase was greatly affected by
the addition to the incubation mixture of such -SH reagents as PCMB and
iodoacetate. As Figure 3 shows, PCMB completely prevents the inhibition of
peroxidase by CoA, and in fact the control level of enzymatic activity is
slightly exceeded in its presence. This suggested that if PCMB were present
from the time of grinding, the spontaneous decay in activity which takes
place could be largely prevented. According a *brei* was prepared using as
grinding medium a buffered solution of PCMB. Table 2 gives the results
of two such experiments, from which it is clear that not only the peroxidase,
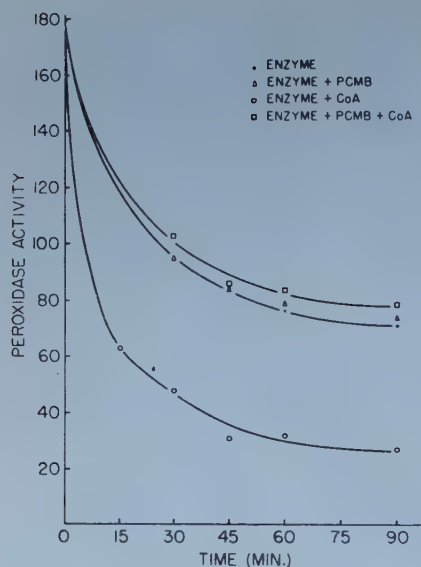
Figure 3.

Figure 4.

Figure 3. *The prevention of CoA — mediated inactivation of peroxidase activity by 10^{-4} M PCMB. Cf. Figure 1.*
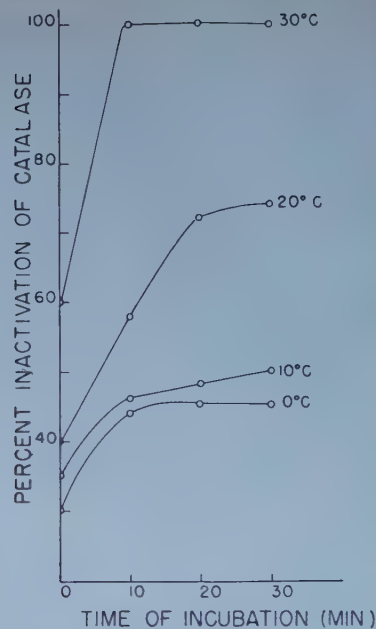
Figure 4. *The effect of temperature on the inactivation of catalase by 5×10^{-4} M CoA.*

Table 1. *Percentage decrease in activity of pea root peroxidase in the presence of sulfhydryl compounds.*

Initial activity — 130 Klett units of purpurogallin color produced in 30 seconds with 0.2 ml. enzyme.

Incubation time	Inhibitor conc.	Inhibitor		
		Coenzyme A	Cysteine	Glutathione
15 min.	0	13	13	13
	1×10^{-5}	28	16	16
	1×10^{-4}	34	24	11
	1×10^{-3}	41	21	21
30 min.	0	31	31	31
	1×10^{-5}	37	29	37
	1×10^{-4}	51	28	25
	1×10^{-3}	53	32	43
45 min.	0	32	32	32
	1×10^{-5}	42	29	30
	1×10^{-4}	67	28	35
	1×10^{-3}	62	24	54

Table. 2. *The effect of addition of 10⁻⁴ M PCMB to the grinding medium on the activity of peroxidase and catalase of pea root brei.*

Peroxidase activity (Klett units) of tissue ground in		Catalase activity (K value) of tissue ground in	
Control Buffer	Buffer and PCMB	Control Buffer	Buffer and PCMB
184	398	.033	.048
125	300	—	—

Table 3. *Percentage decrease in activity of crystalline beef liver catalase incubated with various sulfhydryl compounds.*

Initial activity, k=0.20; incubation time, 45 min., temperature=30° C. Control showed no spontaneous inactivation during this period.

Inhibitor conc. M	Inhibitor		
	Coenzyme A	Cysteine	Glutathione
1×10^{-5}	38	19	43
1×10^{-4}	55	28	41
1×10^{-3}	72	35	30

but also the catalase activity is greatly enhanced by the incorporation of PCMB into the grinding medium.

2. Inactivation of crystalline catalase.

It was considered desirable to ascertain whether coenzyme A could produce its effect on a crystalline enzyme in the absence of other cellular components. Because of its ready availability, crystalline catalase was chosen for these studies. Catalase was incubated with CoA or other -SH compound and activity of aliquots of the reaction mixture measured at intervals. Table 3 shows the results of an experiment in which CoA, glutathione, and cysteine were incubated separately with catalase at three different concentration levels. Once again it is apparent that CoA inhibits much more effectively than either of the other two compounds.

Coenzyme A was similarly found to inactivate the peroxidase and tyrosinase of radish roots, the peroxidase of cucumber fruits, and the tyrosinase of *Drosophila* (kindly furnished by Dr. M. Fling).

The effect of temperature on the inactivation of catalase by CoA was determined. It is clear from the data of Figure 4 that the reaction proceeds more rapidly as the temperature is raised from 0—30° C, with a Q_{10} approaching 2.

Discussion

The inhibition of enzymes of the hydroperoxidase group by sulfhydryl compounds has been previously noted by various workers (3, 5, 7), and it has been suggested (3) that the union occurs *via* the iron of the enzyme. The phenomena described in this paper appear to resemble these previous reports except that coenzyme A is much more effective than any of the other -SH compounds reported by previous workers. The spontaneous decline in activity of peroxidase of pea root *brei*, while not previously reported, finds a parallel in the slow inactivation of catalase of *breis* of *Mytilus californicus* (6), attributed to the presence of endogenous glutathione. The fact that PCMB added during preparation (Table 2) greatly reduces the spontaneous inactivation of peroxidase indicates that sulfhydryl groups are mainly responsible for this phenomenon in pea *brei*. It does appear, however, that this spontaneous decline in activity cannot be entirely prevented by PCMB (Figure 3), leaving open the possibility that other types of compounds are involved in this reaction. Another uncertainty of interpretation is occasioned by the fact that PCMB added after grinding is without effect on the subsequent spontaneous inactivation of peroxidase, yet PCMB added at any time inhibits the CoA-mediated inactivation.

Although CoA inhibits in concentrations markedly lower than either glutathione or cysteine, the concentrations required are still so great as to preclude its usual mode of catalytic action. Although the inhibition of peroxidase by CoA is probably not of physiological significance, no definite statement can be made in the absence of data on the relative amounts of enzyme and CoA in the tissue.

Summary

1. The peroxidase activity of pea root *brei* declines rapidly immediately after grinding. This spontaneous inactivation is accelerated by coenzyme A in concentrations as low as 10^{-5} M. Glutathione is also effective but much higher concentrations are required to cause a comparable degree of inactivation. The catalase and tyrosinase activities of various plant *breis* are similarly affected. These inhibitions are completely prevented by PCMB.

2. Crystalline catalase is similarly inactivated by CoA in the absence of other addenda. The inactivation process is markedly temperature-dependent, with a Q_{10} approaching 2.

3. The addition of 10^{-4} M PCMB to the phosphate buffer in which the tissue

is ground prevents the spontaneous inactivation, and frequently yields *breis* of significantly higher enzymatic activity than controls without PCMB. This fact may be of general significance for the problem of quantitative estimation of enzymatic activity in plant *breis*.

References

1. Chance, B.: The enzyme-substrate compounds of bacterial catalase and peroxides. — Biochem. Jour. 46: 402. 1950.
2. Galston, A. W.: Some metabolic consequences of the administration of indoleacetic acid to plant cells. — Proc. 3rd International Plant Growth Substance Symposium, Wye College, England (in press). 1956.
3. Keilin, D. & Hartree, E. F.: On the mechanism of decomposition of hydrogen peroxide by catalase. Proc. Roy. Soc. London 124 B: 397. 1938.
4. Lemberg, R. & Legge, J. W.: Hematin compounds and bile pigments. — International Publishers, Inc. New York. 1949.
5. Lipmann, F.: The oxidation of p-aminobenzoic acid catalyzed by peroxidase, and its inhibition by sulfanilamide. — Jour. Biol. Chem. 139: 977. 1941.
6. Marks, G. W.: The effect of glutathione and other substances on the inactivation of catalases. — Jour. Biol. Chem. 115: 299. 1936.
7. Stern, K. G.: Über die Hemmungstypen und den Mechanismus der katalatischen Reaktion. — Z. f. phys. Chemie 209: 176. 1932.
8. Sumner, J. B. & Somers, G. F.: Chemistry and Methods of Enzymes. — Academic Press, New York. pp 180—181. 1943.

Chemical Factors Limiting Lateral Root Formation in Isolated Pea Roots

By

JOHN G. TORREY

Department of Botany, University of California,
Berkeley, California
(Received April 9, 1956)

Introduction

Seedling root morphogenesis depends fundamentally upon the spatial distribution of cellular divisions along the axis of the elongating primary root and the orientation of divisions with reference to that axis. A small proportion of the total number of cell divisions which occur during root development takes place specifically in the pericycle opposite the protoxylem points of the primary xylem tissue and these divisions are oriented in the radial direction. Each such periclinal cell division with its immediately associated subsequent cell divisions represents the initial step in the formation of a lateral root primordium. The morphogenesis of the branched form of a tap root system depends upon the site along the root axis of these initial divisions.

In an earlier paper (Torrey 1950) the factors controlling lateral root formation in isolated pea roots were studied. Indoleacetic acid (IAA) was found to be required for the initiation of cell divisions leading to lateral root formation. In addition, evidence was presented which suggested that an unidentified factor acted together with auxin in the initiation of lateral root primordia. The factor was believed to move from the cotyledons into the elongating seedling root. Under experimental conditions such as decapitation of isolated roots in culture, it could be shown to move in an acropetal direction.

Recently, Geissbühler (1953) using cultured roots of *Vicia faba*, studied further the process of lateral root initiation, demonstrating the importance of the presence of the cotyledons for normal lateral root formation in the early development of the seedling root. Lateral root initiation was found to become independent of the cotyledons as the root developed. Geissbühler emphasized the constancy of the distance between the primary root tip and the point at which lateral roots first appear along the length of the root, a relationship which he interpreted as due to the inhibitory effect of the root tip itself. Geissbühler found, in general, a positive correlation between root elongation and the number of lateral roots formed but was unable to study separately the factors influencing primary root elongation and those controlling lateral root formation.

Under the cultural conditions used in earlier experiments (Torrey 1950), it was found that pea root tips excised from the germinating seed and grown through one subculture for one week were apparently depleted of the factors necessary for lateral roots. The present paper is devoted to a study of the chemical factors controlling lateral root initiation in these first transfer root tips. Evidence is presented indicating that a number of known growth factors may limit lateral root initiation under different cultural conditions and that, in addition, the apical meristem of the root tip may determine root morphology by producing an inhibitor of lateral root initiation.

Materials and Methods

Cultural procedures essentially similar to those described in earlier work (Torrey 1950) were used. Seeds of the garden pea. *Pisum sativum*, variety Alaska, (Asgrow strain), were used throughout. Five mm root tips from seeds germinated for 48 hr. in sterile distilled water were excised and grown on nutrient medium in 11 cm. Petri dishes for one week in the dark at 25° C. These primary root tips were grown for the first week on a modified Bonner agar medium (Torrey 1954) containing only major salts, iron, and sucrose but lacking vitamins and other trace elements. The incomplete modified Bonner medium contained per liter of solution: 242 mg. $Ca(NO_3)_2 \cdot 4H_2O$; 42 mg. $MgSO_4 \cdot 7H_2O$; 85 mg. KNO_3; 61 mg. KCl; 20 mg. KH_2PO_4; 1.5 mg. $FeCl_3$; 40 gm. sucrose and 5 gm. Difco Bacto-agar. This same medium was supplemented in various ways during the course of the experiments. All growth factors and other reagents tested were sterilized by cold Seitz filtration and were added to the medium after autoclaving. The pH of the medium was adjusted as indicated in individual experiments after autoclaving by dropwise addition of sterile solutions of 0.05 N HCl or 0.05 N KOH. Manipulation of root tips and segments was carried out in an ultra-violet sterilized transfer room with minimum exposure of the root tissues to daylight (blue) fluorescent lights; roots were then cultured during treatment in continuous darkness at 25° C.

All experiments to be reported here were performed with 10 mm. first transfer

Figure 1. *Five mm. pea root segments cleared in formalin-acetic acid-alcohol fixative after 5 days treatment in the dark on modified Bonner pea root medium with IAA at 10⁻⁵ M.* Top: 5 mm. tip segment, showing localization of 2 lateral roots in middle of segment. A third lateral root is at the same level on the opposite side of the root. Middle: 5 mm. basal segment showing four lateral root primordia distributed along the length of the segment. Bottom: 5 mm. basal segment showing no lateral root primordia after treatment. All ×8.

tips excised from isolated roots which had been grown from 5 mm. primary tips cultured in nutrient medium for one week. The week-old roots prior to tip excision varied in average length from 55 to 60 mm. In early experiments, unsegmented 10 mm. first transfer tips were studied for lateral root initiation; in later experiments, the 10 mm. tips were cut into two 5 mm. segments and the responses of the 5 mm. tips and 5 mm. basal segments were studied separately. In none of the root segments used for this study were there pre-existing root primordia present prior to the treatment, and thus all lateral roots formed were newly initiated structures.

Root tips and segments to be studied for lateral root initiation were killed at the end of the experimental period in formalin-acetic acid-alcohol fixative with aspiration which served to preserve the material and to clear the tissues so that detailed counts of lateral root primordia could be made using a dissecting microscope at 30× magnification with transmitted light. Even very small primordia are distinguishable as dark, discrete, meristematic areas in the cleared material (Figure 1) so that accurate counts can be made using this procedure. For each experimental treatment, 16 to 20 root tips or segments were used. The use of cleared material for lateral root initiation studies has two major advantages over earlier methods. First, a count of macroscopically visible roots requires that sufficient time be allowed subsequent to root initiation for elongation of the laterals to proceed. With cleared materials, this experimental period may be substantially reduced. Second, lateral root initiation is a process distinct from lateral root elongation and is affected differently by different environments. Conditions which favor initiation of laterals may prevent their subsequent elongation. Use of cleared material assures that all lateral roots initiated by a given treatment can be determined.

Experimental Results

At the outset it became apparent that the number of lateral roots initiated per 10 mm. tip depended largely on the nutrient medium in which the tips were maintained during the period of initiation. A number of factors were found to limit lateral root initiation in intact tips, each factor influencing the

effectiveness of the others. These factors include the chemical structure and concentration of the auxin supplied, the duration of auxin treatment, the availability of vitamins and other growth factors, and the pH of the medium. In order to obtain maximum root initiation, every factor must be at its optimum. After establishing what appeared to be the optimum conditions for each of the above factors by a series of experiments in which each factor was varied separately, experiments were made to test the importance of each factor under other otherwise optimum conditions.

A. **Effectiveness of Auxin Treatment**

1. *Chemical structure and concentration*: — Although no extensive survey of effectiveness of different auxins was made, several auxins were tested over a range of concentrations. In Table 1 are summarized the results of these experiments. These tests were carried out with 5 mm. basal root segments cultured on a complete nutrient medium at optimum pH. It is clear that over the range of concentrations tried, IAA was the most effective auxin tested in inducing lateral root formation. In pea roots, IAA is about $100\times$ more effective on a molar basis than the related nitrile, IAN. In addition to causing relatively poor root initiation, IAN was likewise ineffective in inhibiting root elongation. Whereas at 10^{-5} M for 5 days, IAA inhibited root elongation by 90 per cent, IAN produced only 22 per cent inhibition: at 10^{-6} M, IAA caused 44 per cent inhibition while IAN produced no inhibition. 2,4-D, although causing almost complete inhibition of root elongation for 5 days at 10^{-5} M, was relatively ineffective in initiating lateral roots and, in addition, caused secondary morphological effects including sloughing of the epidermis. On the basis of these and similar experiments, IAA was selected as the auxin to be used in subsequent studies on root initiation in pea roots.

Table 1. *Lateral root formation in 5 mm. basal root segments cultured for 5 days in the dark on a modified Bonner agar pea root medium containing auxin as noted below, 0.1 mg/l thiamin HCl, 0.5 mg/l nicotinic acid and added trace elements at pH 6.1—6.2.* Average number of laterals per ten basal root segments and standard errors.

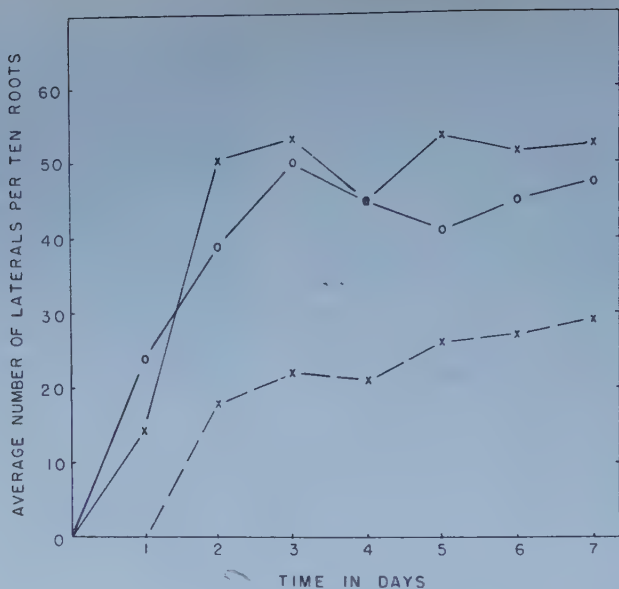| Auxin	Auxin Concentration (Molarity)			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Indoleacetic acid (IAA)	26 ± 5.2	57 ± 3.4	29 ± 2.7	15 ± 2.2
Indoleacetonitrile (IAN)	—	17 ± 2.7	10 ± 1.2	9 ± 1.5
α-Naphthaleneacetic acid (NAA)	25 ± 4.8	32 ± 4.7	29 ± 4.4	--
2,4-dichlorophenoxyacetic acid (2,4-D)	12 ± 3.4	21 ± 5.3	26 ± 6.3	—

Figure 2. *The effect of duration of auxin treatment on lateral root formation in unseg-mented 10 mm. first transfer pea root tips.* IAA concentration at 10^{-5} M. Circles: average number of laterals formed per 10 roots after IAA treatment for various periods followed by transfer to auxin-free medium until the seventh day. Crosses: average number of laterals per 10 roots after IAA treatment for various periods followed by immediate fixation; solid line — average total number of laterals formed on entire root; broken line — average number of laterals formed in the 5 mm. tip portion only. Note the lag in initiation of laterals on the tip half as compared to the basal half.

From Table 1 it is evident that, over the concentration range tested, IAA showed greatest root initiating activity at 10^{-5} M At higher concentration, the effect of the auxin was one of toxicity; at lower concentration, the root-initiating activity decreased. As in previous studies (Torrey 1950, 1952), lateral root formation and root elongation as a function of auxin concentration showed an inverse correlation.

2. *Duration of auxin treatment*: — Several experiments with unsegmented 10 mm. first transfer root tips were made to determine the period of exposure of roots to auxin-containing medium to induce a maximum response of root initiation. Two types of experiments were performed. In both experiments a complete nutrient medium was used. In one treatment, roots were placed on IAA-medium for periods varying from 1 to 7 days. At the end of each 24-hr. period, a set of root tips was removed and the roots were killed in fixative solution. In the other type of experiment, roots were also placed on IAA-medium for different periods, varying from 1 to 7 days However, at

the end of the auxin treatment, roots were transferred to the complete medium lacking auxin and then allowed to continue to develop until the 7th day when all roots were fixed at one time. In the latter treatment, induced lateral roots were able to elongate during the period on auxin-free medium.

In Figure 2 are presented the results of these two experiments. The two methods gave essentially the same result. As in earlier experiments with initial tips (Torrey 1950), 3 days of treatment gave maximum response to the auxin. The total number of lateral roots initiated remained fairly constant with further auxin treatment. Laterals in the tip half of the root were delayed in their formation, first appearing after the second day. A period on control medium after auxin treatment did not further increase lateral root initiation. It should be noted that these counts, determined on cleared roots, are of lateral root primordia which were, for the most part, not evident in the external morphology of the root. From these experiments an arbitrary period of auxin treatment of 5 days was chosen, which was followed by immediate killing of tissues in fixative solution.

B. Lateral Root Initiation in Intact Versus Segmented Ten Millimeter Tips

In preliminary experiments it became evident that the number of lateral roots initiated per 10 mm. tip depended not only upon the constitution of the nutrient medium in which the tips were maintained during the period of initiation, but also upon the physical relationship between the root meristem region and the remainder of the root segment. A number of lines of evidence, some of which will be discussed later, pointed to the idea that the root meristem has an inhibitory effect on lateral root initiation in the region of the root proximal to the root apex. A series of experiments was performed which confirmed this view. The experiments involved the simple procedure of dividing excised 10 mm. root tips into two segments prior to treating with auxin. In each series, roots were cut into basal and tip segments of different lengths. Thus, in one series, roots were cut in half, i.e., into 5 mm. bases and 5 mm. tip segments, with a basal segment to tip segment length ratio of $5:5$. In other series, roots were cut so that basal length to tip length ratios were $6:4$, $7:3$, $8:2$, and $9:1$, respectively. Root segments were placed on the complete nutrient medium containing 10^{-5} M IAA at pH 6.2 for 5 days and then all segments were fixed and counts of lateral roots were made. The design of the experiment and the results are shown in Figure 3.

Intact roots responded to the complete medium as before; approximately half of the laterals were initiated in the tip 5 mm. portion of the root and
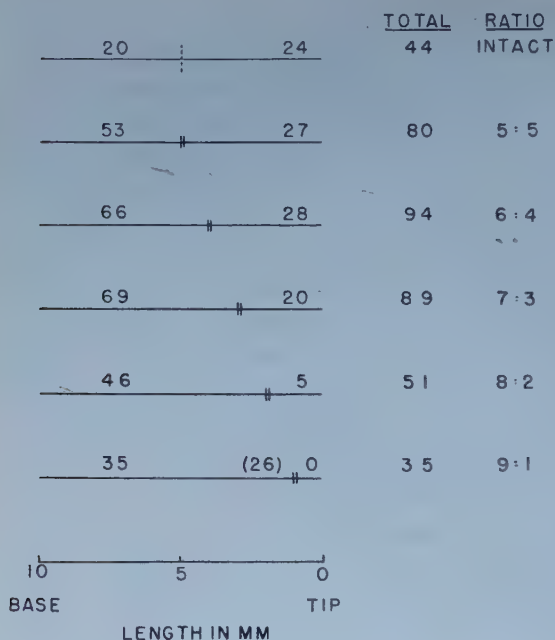
AVERAGE NUMBER OF LATERALS PER TEN ROOTS

		TOTAL	RATIO
20	24	44	INTACT
53	27	80	5 : 5
66	28	94	6 : 4
69	20	89	7 : 3
46	5	51	8 : 2
35	(26) 0	35	9 : 1

```
10          5          0
BASE                   TIP
   LENGTH IN MM
```

Figure 3. *Lateral root formation in segmented 10 mm. first transfer root tips cultured for 5 days on complete modified Bonner medium containing 10^{-5} M IAA. Diagram of root in each series shows point of segmentation (double vertical lines) with the number above each segment representing the average number of laterals per 10 root segments. Top line represents unsegmented root with the distribution of laterals in basal and tip halves indicated.*

the rest along the length of the lower half. However, when the 10 mm. tip was cut in half and treated in the same medium, there was a marked increase in lateral root initiation in the basal half, with the total number of laterals for the two segments increased correspondingly. As the basal segment was increased in length, the number of basal laterals increased until the segment ratio was 7 : 3. Any further increase in basal length resulted in a lowering of lateral root initiation on the basal segment. Lateral root initiation in roots with 1 to 2 mm. tip segments excised was very reduced, because the excision occurred in the region where tip laterals are usually formed.

From these experiments, it appears that lateral root initiation in the basal portion of the intact 10 mm. tip is prevented by an inhibitory effect from the region of the root tip. Separation of the basal segment from the tip removes this inhibition. Study of the results with 3, 2, and 1 mm. root tip excision suggests that the inhibitory effect resides in the 2.0 mm. tip, the morphological region which includes the apical meristem and the elongating region immediately proximal to it.

As a standard experimental procedure for subsequent studies of lateral root initiation, 10 mm. first transfer root tips were cut into two 5 mm. segments and usually both basal segments and tip segments were transferred

to the test medium, widely separated on the agar surface in a Petri dish. After 5 days of treatment in the dark, the roots were fixed and counts of lateral root primordia were made on the cleared roots. The basal segments show no elongation whether treated with auxin or not; tip segments are inhibited almost completely in their elongation by the auxin concentrations used. This procedure, which was finally adopted for a careful analysis of the chemical factors controlling lateral root initiation in pea roots, has several advantages over earlier test methods and other published methods. In the first place, lateral root initiation in 5 mm. basal segments occurs independent of root elongation which has usually been a complicating factor in such studies. The basal segments include only fully elongated, although not completely mature, tissues. Lateral root initiation can thus be sharply separated from the process of root elongation. In the second place, 5 mm. basal segments are apparently quite dependent on the nutrient medium for their lateral rooting capacity and thus the nutritional requirements for lateral root initiation may be carefully assessed under aseptic conditions. In the third place, the complication of root tip inhibition of lateral root initiation is removed and can itself be studied independently. Finally, the total number of laterals initiated is sufficiently large to give statistically significant data subject to careful analysis.

C. Growth Factor Requirements for Lateral Root Initiation

Since it was recognized that lateral root initiation in excised pea root tips depended to a large extent on factors supplied to them via the nutrient medium, it was desirable to deplete the root materials to be used for tests of endogenous growth factors. It was for this reason that 5 mm. root tips excised from the germinating seed were grown for one week in a medium containing only the macronutrient elements, iron and sucrose, but lacking growth factors and other trace elements. While root elongation in this incomplete medium was consistently good, averaging 55 to 60 mm., apparently such roots use up reserve growth factors carried over from the seed, so that the growing tips are essentially dependent upon the external medium for their supply. Ten mm. tips were excised on the seventh day, either cut into two 5 mm. segments or transferred intact to different media to which various growth factors had been added, either individually or in different combinations. Typical results of these experiments are presented in Table 2.

From these experiments one is led to conclude that several specific chemical factors contribute, either directly or indirectly, to the process of lateral root initiation. Auxin activity is apparently of primary importance; in its

Table 2. *Lateral root formation in segmented and intact 10 mm. pea root tips cultured for 5 days in the dark on incomplete modified Bonner medium with various supplements at pH 6.0—6.5.* Trace elements include 1.5 mg/l $ZnSO_4$, 4.5 mg/l $MnSO_4$, 0.25 mg/l $Na_2MoO_4 \cdot 2H_2O$, 1.5 mg/l H_3BO_3, and 0.04 mg/l $CuSO_4 \cdot 5H_2O$. Average number of laterals per ten root segments and standard errors.

A. Segmented ten mm roots			
Medium	Segment		Total
	Basal	Tip	
Control (macronutrient salts, iron and sucrose)	3 ± 1.6	0	3
Control$+10^{-5}$ M IAA	2 ± 1.8	14 ± 1.8	16
Control+IAA+0.5 mg/l nicotinic acid (NA)	13 ± 3.7	16 ± 1.5	29
Control+IAA+0.1 mg/l thiamin HCl (T)	33 ± 3.9	19 ± 1.5	52
Control+IAA+42 mg/l adeninesulfate (A)	11 ± 4.1	29 ± 0.6	40
Control+IAA+trace elements (EL)	19 ± 5.2	11 ± 2.2	30
Control+IAA+NA+T	41 ± 3.4	23 ± 2.2	64
Control+IAA+NA+T+EL	55 ± 2.1	24 ± 1.5	79
Control+IAA+NA+T+EL+A	67 ± 3.8	35 ± 2.0	102

B. Unsegmented ten mm roots			
Medium	Region		Total
	Basal	Tip	
Control	0	0	0
Control+IAA	0	13 ± 2.8	13
Control+IAA+NA+T+EL+A	36 ± 3.9	26 ± 1.9	62

absence, no root initiation occurs. The B vitamins, thiamin and nicotinic acid, increase the response of the basal segments to auxin treatment with the greater effect being elicited by thiamin. The effects of the two are apparently additive. Nicotinamide serves equally as well as nicotinic acid. A slight but consistent improvement was evident upon addition of the trace elements in the presence of the vitamins and auxin. Addition of adenine to this medium resulted in another significant increase in the number of laterals produced. This effect was greatest in the 5 mm. tip. The highest total number of laterals on the two segments together was produced by this last treatment, which is referred to henceforth as the "complete" nutrient medium.

In the intact 10 mm. tip, the response is essentially similar (Table 2 B), although the total number of laterals formed is lower, due apparently to the inhibitory effect of the root apex. It is interesting to note that auxin by itself stimulates lateral root initiation in the region of the root immediately behind the swollen tip, where the laterals all arise in a ring at one level about 2 to 3 mm. from the tip (Figure 1). This tip response to auxin alone is also found in segmented roots. Laterals are initiated in the basal half of the intact root

only when the growth factors are supplied in the medium. This fact suggests that lateral root initiation in apical and in basal segments requires somewhat different conditions, with the tip laterals less dependent on the external medium than basal laterals. The lag between tip and basal lateral root initiation observed in Figure 2 is also evidence of a difference in behaviour. The only compound tested which was found to increase significantly the initiation of laterals by 5 mm. tip segments was the compound adenine. Lateral roots initiated in 5 mm. basal segments showed no apparent polar distribution as reported by Nagao (1942) and as occurred in decapitated pea roots (Torrey 1950). Apparently, an adequate supply of chemical factors in the nutrient medium overcame the internal polar distribution which limited lateral root initiation.

A large number of additional vitamins and growth factors, amino acids, and other organic compounds tested for root initiation activity in the root segment test were found to have little or no activity in the range of concentrations tried. Compounds found inactive when tested in the presence of 10^{-5} M IAA in the complete medium described above include: pyridoxine HCl, folic acid, ascorbic acid, thioctic acid, glycine, cysteine, glutamic acid, thioglycollic acid, arginine, glutathione, 6-furfurylamino purine, and coconut milk. This list includes many of the compounds reported in various tests to show a stimulating effect on cell divisions following auxin treatment.

D. The Effect of pH on Lateral Root Initiation

In a study of the nutritional requirements for growth of the apical meristem of the primary root of peas (Torrey, 1954), it had been observed that the pH of the medium was critical in determining root elongation, showing an optimum for elongation around pH 5.0. In view of the marked effect of pH on meristematic activity of half mm. pea root tips, a study was made of the effect of different hydrogen-ion concentrations on lateral root initiation. The complete nutrient medium described above was used and tests were run with 10 mm. root tips cut into two 5 mm. segments. Measurement of the pH of the medium was made at the beginning of the experiment and again at the end of the treatment period by immersing the electrodes in a sample of the cold agar medium. No significant change in the pH of the medium occurred during the 5-day treatment.

In Figure 4 are summarized graphically the combined results of several experiments in which the pH of the medium was varied from 4.0 to 8.4. The numbers of laterals produced on tip segments and on basal segments are plotted separately. It is evident that over much of the pH range tested,
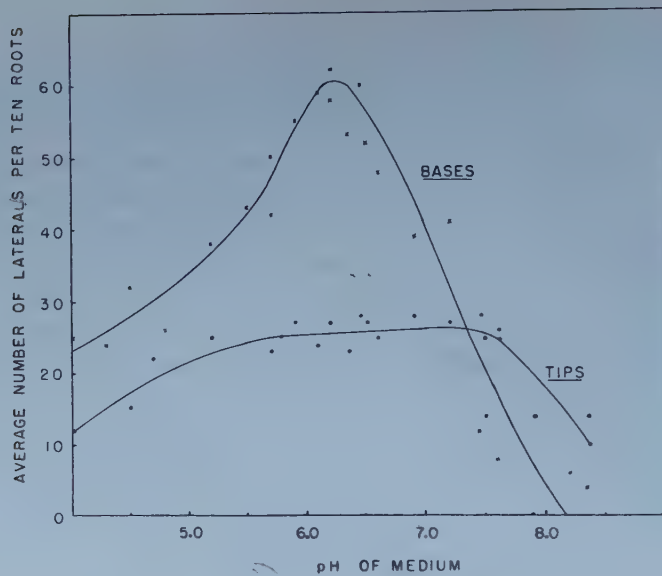
Figure 4. *The effect of pH on lateral root formation in segmented 10 mm. first transfer root tips cultured for 5 days on complete modified Bonner medium containing 10^{-5} M IAA. Laterals on basal 5 mm. segments (crosses) and tip 5 mm. segments (dots) are plotted separately.*

lateral root formation by tip segments is relatively constant and more or less unaffected by pH changes. In basal segments, on the contrary, there is a marked effect of pH on the numbers of laterals formed; the initiation process shows an optimum curve with a peak in the pH range from 6.0 to 6.4 and sharp declines on either side of this optimum. At pH 8.0 almost no laterals are initiated.

In similar tests with intact 10 mm. root tips, essentially the same responses to pH variations were evident, *i.e.*, tip laterals were formed at a fairly constant number regardless of the pH of the medium between pH 4 and 8, while increasing numbers of laterals were formed in the 5 mm. basal half of intact tips in response to increasing pH's up to about pH 6.5. It was quite apparent that changes in pH affected most markedly the initiation of basal laterals. These same basal laterals, both in intact and in segmented roots, have already been shown to be dependent upon the growth factors supplied in the medium and to be inhibited by the presence of the tip.

A number of attempts were made to overcome the inhibitory effects of the high pH by manipulation of the medium. Boll and Street (1951) have shown that the depression of tomato root growth *in vitro* due to a high pH level

of the medium could be overcome by supplying iron in a chelated form, either as the tartrate or as Fe ethylenediamine-tetraacetic acid (EDTA). Providing pea root segments with chelated iron in either of these forms had no effect on inhibition of lateral root initiation at pH 7.0 or greater. According to Burström (1952, 1953) many pH effects in root growth phenomena are attributable to effects on calcium availability. Manipulation of the calcium levels in the complete medium over a wide range of concentrations showed no effect on lateral root initiation. Audus (1949) suggested that auxin dissociation accounts for the varying response of roots to pH. Increasing auxin concentrations at pH's of predicted low auxin dissociation did not increase lateral root initiation in the root segment test used here. Use of nicotinamide in place of the free acid over a range of pH's to avoid permeability limitations due to the dissociation of the molecule (see Ihm 1954) did not change the response to H-ion concentration. Clearly, pH plays an important role in controlling root morphogenesis *in vitro*; no explanation of the pH effect is at present evident. Presumably, the hydrogen-ion concentration of the medium determines the effective concentration at the site of root initiation of one or more metabolites essential for the processes of cell division and primordium formation.

E. **Inhibition of Lateral Root Initiation**

1. *Antimetabolites*: — In order to demonstrate unmistakably the involvement in the initiation process of growth factors provided in the nutrient medium, two methods may be used. In one procedure, it should be possible to deplete the tissue of a factor which it requires for the process but is incapable of synthesizing itself or synthesizes at a limiting rate. In the complete absence of the factor no initiation will occur; upon addition of the factor, initiation will proceed. In this method one establishes a deficiency of the factor which can be remedied by its addition to the medium. The results of this type of approach have been described above. The second method involves the use of a specific antimetabolite, i.e., a chemical analogue of a growth factor, which competitively inhibits the action of the factor in its usual physiological role. This latter method has been used to substantiate the results of the first method for demonstrating the growth factor requirements for lateral root initiation in pea root segments.

If, in fact, the vitamins thiamin and nicotinic acid and the purine adenine are specifically required for the initiation process, as is suggested by the above nutritional study, one should be able to decrease or prevent lateral root initiation by competitively inhibiting the action of these growth factors

Table 3. *Lateral root formation in segmented 10 mm. pea root tips cultured for 5 days in the dark on complete medium lacking added adenine with IAA at 10^{-5} M, pH 6.0—6.5. Metabolites and antimetabolites added as indicated. Average number of laterals per ten roots and standard errors.*

Concentration of Antimetabolite (mg/l)	Concentration ef Metabolite (mg/l)	Basal segment	Tip segment	Total
Pyrithiamin	*thiamin*			
0	0.1	29 ± 4.1	19 ± 1.7	48
0.1	0.1	28 ± 5.0	17 ± 2.4	45
1.0	0.1	18 ± 2.1	15 ± 3.8	33
5.0	0.1	3 ± 2.1	1 ± 1.1	4
3-acetylpyridine	*nicotinic acid*			
0	0.5	23 ± 5.8	21 ± 2.6	44
1	1	22 ± 5.7	9 ± 2.0	31
10	1	17 ± 5.7	10 ± 1.7	27
20	1	12 ± 3.6	1 ± 0.9	13
10	5	27 ± 4.2	12 ± 2.1	39
10	10	39 ± 4.1	8 ± 1.7	47
2.6-diaminopurine	*adenine*			
0	0	29 ± 1.8	7 ± 1.4	36
0.1	0	29 ± 4.1	10 ± 2.6	39
1	0	10 ± 2.7	4 ± 1.2	14
5	0	10 ± 3.0	0	10
1	42	47 ± 2.7	17 ± 1.8	64
1	84	56 ± 4.2	19 ± 2.2	75

with their chemical analogues. Experiments were performed in which the effectiveness of the specific antimetabolites was determined over a range of concentrations in the presence of the growth factor itself.

In Table 3 are presented typical results of experiments with these three antimetabolites. Although the data are not extensive enough to demonstrate conclusively that each of these antimetabolites is a competitive inhibitor of the particular metabolite, it is clear in each case that the presence of the antimetabolite progressively decreases lateral root initiation with increasing concentration and that adding the growth factor restores the capacity of the root segment to initiate lateral root primordia, a restoration which is directly related to the increasing concentration of the essential metabolite. These data give support to the view that under given conditions, each of the three factors may limit lateral root initiation. It is interesting that at a sufficiently high antimetabolite-metabolite ratio, lateral root initation in both tip and basal segments is inhibited, suggesting that all of these three factors are essential to the process in both tip and basal segments.

2. *A natural inhibitor of lateral root initiation*: — The experiments with segmented roots (Figure 2) suggested strongly that lateral root initiation in the basal half of 10 mm. root tips is normally inhibited by the apical half

Table 4. *Effect of a natural inhibitor of lateral root formation removed by ether extraction from pea root tips.* Extract added to complete modified Bonner medium with IAA at 10^{-5} M at pH 6 2. Intact and segmented 10 mm pea root tips cultured 5 days in dark. Average number of laterals per ten roots and standard errors.

Material	Basal laterals	Tip laterals	Total
Exp. 1. Unsegmented 10 mm root tips			
Control	29 ± 4.2	19 ± 1.3	48
Control+1×ether extract	9 ± 3.3	16 ± 1.9	25
Control+0 1×ether extract	13 ± 3.0	24 ± 1.9	37
Exp. 2. Basal 5 mm segments only			
Control	47 ± 3.1	—	
Control+1×ether extract	23 ± 4.4	—	
Control+0.1×ether extract	39 ± 2.6	—	
Exp. 3. Segmented 10 mm root tips			
Control	33 ± 5.0	16 ± 2.2	49
Control+1×ether extract	14 ± 2.4	6 ± 1.6	20
Control+0.1×ether extract	34 ± 4.0	14 ± 1.5	48

of the root. There have been a number of suggestions in the literature that the root tip inhibits lateral root initiation in the region of the tip and immediately proximal to it, accounting for a fairly constant minimal distance between the tip and the first lateral roots behind the tip (Fries 1954, Geiss-bühler 1953, Nutman 1952). Because of the convenience of the experimental materials used here, an attempt was made to demonstrate the inhibition of lateral root formation in basal segments by extracts of pea root tips.

The following extraction procedure was used. Five hundred 5 mm. root tips were excised from 48 hr. aseptically germinated pea seeds and were immersed directly in an equal volume of diethyl ether in a 125 ml. Erlenmeyer flask which was corked and stored in the refrigerator at $2°$ C. for three days. Then the ether extract was decanted, the root tissues were rinsed with a second equal volume of ether which was combined with the first. The ether extract was evaporated to dryness under vacuum, taken up in 4 ml. distilled H_2O, cold sterilized through a membrane filter and 1 ml. (1×) or 0.1 ml. (0.1×) aliquots were added to 4 ml. of the complete nutrient medium which included 10^{-5} M IAA. Variations of this procedure, including alkaline and acid extraction were also used. In certain experiments, 3-day extraction in sterile, distilled water was carried out and then ether extraction of the aqueous supernatant was made.

Tests of the effectiveness of the extracts were made on intact 10 mm. root tips and on the 5 mm. tip and basal segments which were cultured on 6 cm. Petri dishes containing only 5 ml. volume of nutrient medium plus extract. In Table 4 are presented results from the two separate types of experiments. It is clear in both the intact roots and segmented roots that the extract added

at its highest concentration had a marked effect on basal lateral root initiation, showing greater than 50 per cent inhibition in all of the experiments cited. Dilution of the extract in general substantially decreased the effectiveness of the inhibition. In a similar experiment, a 3-day aqueous extract of root tips, extracted in turn with an equal volume of ether and handled as described above, produced an equivalent inhibition of basal laterals. The number of tip laterals initiated, although consistently reduced by the treatment with higher extract concentration, was less affected than the lateral initiation in the basal segments. Preliminary experiments, made on the solubility properties of the inhibitory material, suggest that ether extraction represents a relatively simple and direct method of obtaining the inhibitory material.

Discussion

In the normal development of the seedling root in Pisum, lateral root formation occurs characteristically in an acropetal sequence. In earlier studies (Torrey 1950, 1952) it was shown that, in addition to auxin, some other factor (or factors) usually provided to the seedling root from the cotyledons is essential for lateral root initiation. In the present study, an attempt has been made to identify the chemical substances required for the lateral root initiation process. In these experiments, it has been demonstrated that cell divisions leading to lateral root initiation in pea root segments can be inhibited or prevented by limiting the supply of any one of the following specific chemical substances: indoleacetic acid, thiamin, nicotinic acid, adenine, and one or more micronutrient elements. In addition, it has been shown that the initiation process is not only affected by limiting supplies of these substances, but also may be prevented by a naturally-occurring inhibitor produced in the root apex.

There are a number of reports of specific substances which influence root initiation in pea stem and pea root tissues. In the root initiation test of Went using etiolated pea stems (Went and Thimann 1937) it has been reported that, in addition to available carbohydrate (sucrose) and auxin (IAA), factors which may limit root initiation include· biotin (Went and Thimann 1937, p. 239), thiamin (Went, Bonner and Warner 1938), adenine (Galston and Hand 1949), nicotinamide and tryptophan (Galston 1949). Fries (1951) reported that arginine influences the position along the length of the primary root at which lateral roots are initiated. In a study of root development in cotyledon-less pea seedlings, Fries (1954) showed that upon the addition of hypoxanthine to the medium the increased root elongation was accompanied by increased lateral root initiation. In these same experiments, arginine had

no effect on the number of lateral roots initiated, while ornithine produced a significant increase in the number of lateral roots formed, a response which also accompanied increased root elongation. In excised pea roots grown in the dark, on the other hand, Fries found that arginine produced an increase in the number of laterals per root. In these studies, it was not possible to distinguish direct effects of these additions upon lateral root initiation since root elongation was invariably affected as well.

In studies of root initiation in plant tissues other than pea, supplementary factors have also been reported to stimulate root formation in the presence of externally supplied auxin. Activity has been attributed to thiamin in cuttings of *Citrus* and *Camellia* (Went, Bonner and Warner 1938), to pyridoxine in cuttings of a number of woody shrubs (Stoutemeyer 1940), to adenine and asparagine in leafy cuttings of *Phaseolus vulgaris* (Thimann and Poutasse 1941), to certain amino acids and purines in cuttings of *Rhododendron* (Doak 1940), to ammonium sulfate and arginine with sucrose in *Hibiscus* (van Overbeek *et al* 1946), to asparagine in detached leaves of *Phaseolus vulgaris* and *Hedera helix* (Gregory and Samantarai 1950), and to vitamin K in *Phaseolus vulgaris* (Hemberg 1953). In all of these cases it has been difficult to relate the action of the supplementary factor directly to the process of root initiation, and the results in certain cases have been interpreted in terms of a beneficial effect on the general nutritional status of the treated plant materials, which was in turn reflected in the rooting response.

In the present studies, lateral root initiation in pea root sections has been shown to depend upon the supply of a number of chemical factors, which are apparently normal requirements for continued metabolic activity leading to cellular divisions in the pericycle. The initiation process can be prevented either by depleting the tissues of any one of these factors or by adding antagonists which prevent the normal metabolic activity of the factor. Lateral root formation can thus be shown to be subject to "multiple-factor control" comparable in some ways to the control of bud initiation in tobacco callus tissue described by Skoog (1953). It is to be expected that under certain experimental conditions of root initiation, one factor may be primarily limiting the initiation process and that the limiting factor may vary as the experimental conditions change, depending on the synthetic capacities or supply within the root. Although in any given situation one essential metabolite may, in fact, limit normal root morphogenesis, the concept of a specific organ-forming substance essential for root formation loses its significance under these varying circumstances. In the light of the multiple-factor control, which is to say the metabolic control, of root initiation in peas, it now becomes possible to understand and unify the many reports of different factors

"controlling" root initiation. Whether or not a particular metabolite will stimulate root formation depends to what extent the metabolite is available in limiting amounts for initiation to proceed.

The present experiments, while implicating a number of possible factors which might limit lateral root initiation in the pea seedling during its normal development, do not provide evidence as to which of these factors is, in fact, provided by the cotyledons to the developing root along a physiological gradient which accounts for the acropetal sequence of root initiation. The careful studies of Dostál (1941) on auxin distribution in pea plants during seedling growth suggest that auxin, together with other factors, moves from the cotyledons into the root to effect lateral branching. It is evident (Torrey 1950, 1952) that essential factors other than auxin are active as well in controlling the morphogenesis of the root. Further experiments on seedling root development will be necessary to determine the natural limiting factors.

The provision of chemical factors from the cotyledons is only one aspect in the control of root initiation in seedling development since the acropetal sequence of root formation is maintained by a balance between the stimulatory effect of chemical factors provided from the cotyledons allowing lateral root initiation at the basal end of the root and the inhibitory action of the tip, preventing the initiation within a given distance from the root apex That the root apex exercises an inhibitory effect on the initiation of new meristems along the axis of the root proximal to the tip is suggested by the morphology of the root itself. Evidence for such an effect has come from the work of Geissbühler (1953) in *Vicia sativa*, is implied in the decapitation experiments of Nutman (1952) on clover roots, and in the work of Fries (1953) on isolated pea embryo development. Howell (1954) found that pea roots contain an ether-extractable substance which inhibits not only epicotyl elongation, but also the initiation of adventitious roots on the etiolated pea stem. This inhibitor is similar in many respects to that reported here. The general concept of an inhibitor of meristem initiation produced in the root apex fits well into the generalizations of Bünning (1952) in which the newly induced lateral root meristems might be considered as "meristemoids" whose initiation along the longitudinal axis of the root is influenced by the meristem of the primary root itself. Further evidence as to the chemical nature of this inhibitor and its action will be essential for a complete understanding of normal root development.

Summary

Using intact and segmented pea roots grown in nutrient culture, a study has been made of the chemical factors limiting lateral root initiation. It has

been possible to demonstrate that, in addition to an auxin, lateral root initiation in pea roots requires an available supply of thiamin, nicotinic acid, adenine and one or more micronutrient elements. Lacking any one of these factors in adequate concentration, cell divisions in the pericycle leading to lateral root formation do not occur. By the addition to the external medium of the antimetabolites pyrithiamin, 3-acetyl pyridine or 2,6-diaminopurine, lateral root initiation can be inhibited; the inhibition is reversed by addition to the medium of the corresponding metabolite in appropriate concentration. The initiation response *in vitro* is dependent upon the pH of the external medium with an optimum response at pH 6.0—6.4 and a very marked inhibition at pH's below 4 or above 8.

Evidence is presented from root segmentation experiments which indicates that the root tip produces a substance which is a natural inhibitor of lateral root formation in pea roots. The inhibitor, extracted by ether extraction from root tips of germinating peas, produces an inhibition of 50 per cent or more of lateral root initiation under otherwise optimum nutritional conditions. The normal acropetal sequence of lateral root formation in intact pea seedling roots is interpreted as the result of a balance between chemical factors moving from the cotyledons toward the root tip and an inhibitor moving from the root tip toward the base of the root.

References

Audus, L. J.: Studies on the pH-relationships of root growth and its inhibition by 2,4-dichlorophenoxyacetic acid and coumarin. — New Phytol. 48: 97. 1949.

Boll. W. G. & Street, H. E.: Studies on the growth of excised roots. I. The stimulatory effect of molybdenum and copper on the growth of excised tomato roots. — New Phytol. 50: 52. 1951.

Bünning, E.: Morphogenesis in plants. — in Survey of Biol. Progress. ed. G. S. Avery. Academic Press, Inc. New York. 1952.

Burström, H.: Studies on growth and metabolism of roots. VIII. Calcium as a growth factor. — Physiol. Plant. 5: 391. 1952.

— Physiology of root growth. — Ann. Rev. Plant Physiol. 4: 237. 1953.

Doak, B. W.: The effect of various nitrogenous compounds on rooting of Rhododendron cuttings with naphthalene-acetic acid. — New Zealand Jour. Sci. Techn. 21: 336 A. 1940.

Dostál, R.: Wuchsstoffstudien betreffend die Korrelationen zwischen Wurzel und Spross bei Pisum sativum. — Acta. Soc. Scien. Nat. Moravicae 13: 1. 1941.

Fries, N.: The influence of amino acids on growth and lateral root formation in cotyledonless pea seedlings. — Experientia 7: 378. 1951.

— Limiting factors in the growth of the pea seedling root. — Physiol. Plant. 6: 292. 1953.

— Chemical factors controlling the growth of the decotylised pea seedling. — Symb. Bot. Upsaliensis 13: 1. 1954.

— Requirements of decotylised pea seedlings cultivated in the dark. — Experientia 11: 232. 1955.

Galston, A. W.: Indoleacetic-nicotinic acid interactions in the etiolated pea plant. — Plant Physiol. 24: 577. 1949.

— & Hand, Margery E.: Adenine as a growth factor for etiolated peas and its relation to the thermal inactivation of growth. — Arch. Biochem. 22: 434. 1949.

Geissbühler, H.: Untersuchungen über die korrelative und hormonale Steuerung der Seiten-wurzelbildung. — Ber. Schweiz. Bot. Ges. 63: 27. 1953.

Gregory, F. G. & Samantarai, B.: Factors concerned in the rooting responses of isolated leaves. — J. Exp. Bot. 1: 159. 1950.

Hemberg, T.: The effect of vitamin K and vitamin H on the root formation in cuttings of Phaseolus vulgaris L. — Physiol. Plant. 6: 17. 1953.

Howell, R. T.: The inhibiting effects of root tips on the elongation of excised Pisum epicotyls cultured in vitro. — Plant Physiol. 29: 100. 1954.

Ihm, P.: Experimentelle Untersuchungen an isolierten Wurzelspitzen. — Zeitsch. f. Bot. 42: 191. 1954.

Nagao, M.: Studies on the growth hormones of plants. V. Polar rootlet formation on root segments cultured under sterile conditions. — Science Reports of the Tohoku Imperial Univ., 4th Series Biology, 17: 137. 1942.

Nutman, P. S.: Studies on the physiology of nodule formation. III. Experiments on the excision of root-tips and nodules. — Ann. Bot., n. s., 16: 79. 1952.

Skoog, F.: Substances involved in normal growth and differentiation of plants. — in Brookhaven Symposia in Biology No. 6, pp. 1—16, Brookhaven National Laboratory, Upton, N. Y. 1953.

Stoutemeyer, V. T.: Vitamin B_6 and the rooting of cuttings. — Amer. Nurseryman 71: 11. 1940.

Thimann, K. V. & Poutasse, E. F.: Factors affecting root formation of Phaseolus vulgaris. — Plant Physiol. 16: 585. 1941.

Torrey, J. G.: The induction of lateral roots by indoleacetic acid and root decapitation. — Amer. Jour. Bot. 37: 257. 1950.

— Effects of light on elongation and branching in pea roots. — Plant Physiol. 27: 591. 1952.

— The role of vitamins and micronutrient elements in the nutrition of the apical meristem of pea roots. — Plant Physiol. 29: 279. 1954.

Went, F. W., Bonner, J. & Warner, G. C.: Aneurin and the rooting of cuttings. — Science 87: 170. 1938.

— & Thimann, K. V.: Phytohormones. — The Macmillan Co., New York. 294 pp. 1937.

Growth and Fruiting Responses of Intact Tomato Plants to Far-Red Radiation

By

S. P. JOHNSON, W. C. HALL and J. L. LIVERMAN

Texas Agricultural and Mechanical College
College Station, Texas
(Received April 10, 1956)

With the onset of conditions of high summer temperature and intense radiation in Texas the vegetative and fruiting processes of commercial varieties of tomatoes become abruptly and drastically curtailed. As a result, potential production is seldom realized. Previous results (5, 6) indicated that partial shading or defoliation would alleviate the adverse effects even under high temperature conditions. The continued accumulation of leaf carbohydrates under summer conditions conducive for high respiration suggested that the curtailment of growth and fruiting involved an auxin-light reaction largely independent of photosynthesis.

An increasing list of light controlled responses, including the flowering of long and short day plants, the germination of light sensitive seeds, stem and coleoptile elongation and leaf expansion of dark grown seedlings, are effectively regulated by energy in the red region of the spectrum and are freely reversible by far-red radiation (1, 2, 3, 9, 10, 11, 12). Liverman and Bonner (9) using Avena sections proposed that the red and far-red reactions involved the auxin-receptor complex with red promoting an auxin receptive entity and far-red decomposing the active auxin complex into an auxin-non-receptive entity.

Previous experiments (6) suggested that the adverse responses of the Marglobe tomato noted under summer conditions in the field could be partly explained in terms of the light-auxin interaction system, particularly the reactions involving far-red. To date no attempt has been made to interpret

the responses of plants growing under natural conditions in the field from this viewpoint.

The experiments reported below show that some of the responses of the Marglobe tomato to natural summer conditions in Texas can be duplicated by far-red irradiation of plants grown in the greenhouse at normal temperatures during the winter months.

Materials and Methods

Marglobe plants were grown in two-gallon jars in fertile soil in the winter months of November through March under conditions considered optimum for growth and fruiting. Night and day temperatures were maintained at 60—65 and 75—85 degrees Fahrenheit. respectively. Relatively few cloudy days were encountered during the experimental period.

Far-red radiation was produced as described by Downs (3). Five 200-watt incadescent-filament lamps, with a filter of two layers of red and two of dark blue cellophane provided the source of far-red energy.

When the plants were approximately 8 inches high, they were divided into two lots. The treatment lot was exposed to far-red radiation (8 a.m.—5 p.m. daily) in addition to winter sunlight. The control lot received sunlight alone. The source of far-red from filtered incadescent lamps was placed initially at 60 cm. above the growing points of the young plants. The plants were then allowed to grow upward toward the far-red source.

Results

Results of earlier work (5, 6) with Marglobe tomatoes show that plants transplanted early in the spring and grown into the high light intensity of summer ultimately become vegetatively and reproductively dormant. Stem elongation was sharply reduced, the petioles and leaflets became twisted and distorted and assumed an acute angle of insertion on the stem or rachis (figure 1 a).

Under far-red irradiation in the greenhouse, similar effects were reproduced (figure1 b). These effects can be compared to the control (figure 1 c) which received normal sunlight alone. The petioles and leaflets grew upward at acute angles; elongation of internodes was inhibited in comparison to the controls. The characteristic effects are shown schematically in figure 2. The far-red effects on petioles and leaflets were primarily localized in the younger tissues formed after exposure and in the adaxial surface of the pulvinoid areas at the base of the leaves or leaflets directly irradiated. The older mature leaves already present prior to exposure showed relatively less effect than

Figure 1. *A comparison of the normal summer-grown plant with irradiated and non-irradiated winter-greenhouse plants.* A. The normal summer-grown Marglobe plant. B. The greenhouse irradiated Marglobe plant. C. The greenhouse non-irradiated Marglobe plant.

those formed after exposure. By removing the blue cellophane and shifting the radiation predominately into the red (5800—7000 Å), the far-red inhibition was reversed, and subsequently formed tissues were normal. The effects obtained with far-red could not be duplicated with ultra-violet although this radiation did cause tissue damage and ultimate death of treated plants.

In the earlier study (6), a high percentage of flowers abscissed as the temperatures increased in the early summer, partly due to high temperature inactivation of pollen. However, late in the summer at the peak of the high temperature period, the number of flowers abscising decreased. To investigate this effect in further detail, four flowering plants were placed 75 cm. from a horizontal far-red source. Temperatures at the stems were approximately one to two degrees higher than room temperature (75—85° F.), but still well below temperatures known to be lethal to Marglobe pollen (90—95° F.) (6). The control plants fruited normally during the experimental period. No abscission of flowers was noted. The results of far-red treatment on flower abscission are shown in Table 1. Far-red treatment initially induced a high rate of abscission of first formed flowers but as flowers developed on later formed spurs there was a definite tendency of the treatment to inhibit abscission. However, the unabscissed flowers did not developed into mature fruits. Following formation of the third fruit spur the unabscissed flowers were sprayed with 10 p.p.m. parachlorophenoxyacetic acid (CPA):
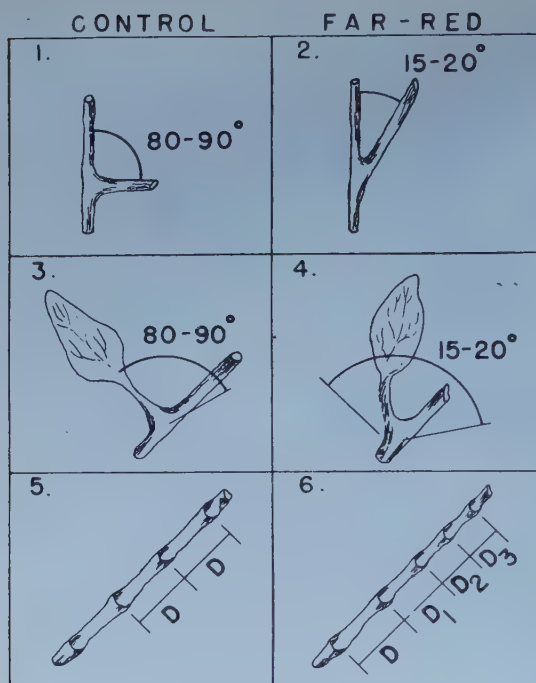
Figure 2. *A schematic presentation of the effects of far-red radiation on petiole, leaflet and stem.* 1. The normal control petiole at an 80—90 degree angle of insertion on the stem. 2. The reduction to a 15—20 degree angle of insertion following far-red treatment. 2 and 3. The similar response of lealfet. 5. The normal non-irradiated stem after elongation. 6. A progressive shortening of internodes following far-red treatment.

all fruits developed but were entirely seedless. Evidently, the far-red radiation had a twofold effect. One effect appears to be inhibition of fertilization possibly by pollen inactivation or reduction in pollen tube growth. A high percentage of pollen grains stained with iodine and observed microscopically were abnormal and gave a negative starch test. Far-red also appears to retard the abscission of unfertilized flowers.

Style exsertion which occurs during the summer in Marglobe flowers was not noted in any of these experiments.

Table 1. *The Effect of Far-red Radiation on Floral Abscission* [1]
in Marglobe tomato.

Spur No.	Average flowers produced	Abscissed	Intact[2]
Spur 1	5	4	1
Spur 2	4	2	2
Spur 3	7	0	7

[1] No abscission occurred in controls during the experimental period.
[2] Young fruits of non-abscissed flowers remained dormant.

Discussion

The results of the present study indicate that most of the adverse vegetative and fruiting responses of Marglobe tomatoes observed during the summer can be duplicated by light treatments involving far-red.

In the tomato, far-red radiation inhibited cell elongation of the main stem resulting in shortened internodes. Far-red differentially inhibited cell elongation of the pulvinoid tissues of petiole and leaflet bases; the faster growth rate on the shaded abaxial side resulted in acute angles of insertion of leaves and leaflets on the stem and rachis.

The inactivation of pollen and the retardation of flower abscission by far-red awaits further clarification. Microscopic examination of pollen from far-red irradiated plants indicated a high degree of non-viable grains. However, normal pollen is high in auxin (14) and the auxins appear to be involved in the abscission of tomato flowers (5). The effect of far-red upon pollen and flower abscission may be mediated through its effect upon the auxin-receptor complex (9).

The far-red responses noted in this study are relatively low energy light reactions, i.e., 100—150 f.c.; hence, high light intensity *per se* is not the sole factor. Further, far-red seems to be most effective at the beginning or end of the photoperiod (7). Earlier experiments (5, 6) showing that shading of plants reverses the growth inhibition of summer sunlight suggest that far-red is most effective in the early morning or late afternoon. Other evidence suggests that the leaves may be involved in the response since removal of leaves of inhibited plants temporarily relieves the radiation effect (6). Since young plants do not show the adverse effect until after some exposure to far-red or to summer conditions (5, 6) it appears likely that a certain level of some inhibitory substance must accumulate before the effect can be observed.

Fritz (4) stated that variations in seasonal irradiation at the earth's surface are known; these are caused by changes in quality and quantity of energy that leave the sun and changes in reflectivity of the atmosphere (including clouds) and the earth's surface. Extensive cloud systems absorb about 20 % and water vapor about 8 % of solar radiation. Although absorbing only about 8 % of total energy, water vapor absorbs the largest amount of solar energy among the atmospheric gases. Fritz (4) further showed an interesting comparison between absorption of the far-red and infrared by precipitable water vapor in the atmosphere: 0.008 cm. water vapor absorbs 68 % of the radiation between 6400 to 7000 Å but only 25 % between 7000 and 8000 Å. whereas 0.082 cm. absorbs 97 % of the radiation in the 6400—7000 Å region, and 62 % of the wavelengths between 7000– 8000 Å. Meteorological data

for the College Station area show that, contrary to popular opinion, more precipitable moisture is in the atmosphere from late spring until late fall than during other parts of the year (over an inch more during July than during January), even though relative humidity is lower during the summer months. The cloud cover is also less during the summer months.

The coinciding of these natural atmospheric conditions with the period of maximum light intensity strongly suggest that the inhibition by summer conditions is caused by a differentially greater absorption in the red region (6500 Å) as compared to the far-red and infrared (7000—8000 Å) regions. Thus the ratio of red to far-red radiation would be greatly altered in summer as compared to the winter months. Swanson et al. (13) studying the effects af near infrared radiation on the chromosomes of Tradescantia, stated that plants exposed to sunlight in the field are subjected to those wave lengths which are believed to be active in inducing the infrared effect and that water vapor in the atmosphere attenuates, but by no means eliminates, solar radiation in the infrared region. The effects of light on the oxidation of IAA and the interaction of red and infrared radiation. therefore, should be given considerable weight in evaluating the causes of summer-induced dormancy in tomatoes. The parallel case of summer-induced dormancy in perennial grasses (8), and in the floral buds and foliage of most deciduous trees as well as a delay in flowering in Okra, Hibiscus esculentus, L.. (7), might also be fruitfully reinvestigated along these lines.

The results of the present study with tomato point up the complexity of the summer-induced dormancy problem and suggest the interrelationship of quality of radiation with other factors in controlling growth and reproduction under these conditions.

Summary

Far-red radiation was found to elicit responses in the Marglobe tomato grown in the greenhouse in winter similar to those observed in Marglobe plants grown under natural summer conditions:

a). Far-red inhibited cell elongation of the stem.

b). Summer induced leaf malformations could be duplicated by far-red radiation of winter-grown plants.

c). Young fruit enlargement was inhibited by far-red.

d). Abscission of first formed flowers was accelerated while abscission of later formed flowers was reduced by far-red.

e. Far-red radiation of flowers resulted in unfertilized fruits.

The qualitative aspects of radiation, particularly red and far-red types of irradiation are believed to be implicated in the summer-induced condition of dormancy.

References

1. Borthwick, H. A., Hendricks, S. B., & Parker, M. W.: Action spectrum for photoperiodic control of floral initiation of a long-day plant, Wintex barley (Hordeum vulgare). — Bot. Gaz. 110: 103. 1948.
2. — — Toole, E. H., & Toole V. K.: Action of light on lettuce seed germination. — Bot. Gaz. 115: 205. 1954.
3. Downs, R. J.: Photoreversibility of leaf and hypocotyl elongation of dark-grown red kidney bean seedlings. — Plant Physiol. 30: 468. 1955.
4. Fritz, Sigmund: Solar radiant energy and its modification by the earth and its atmosphere. — In Comp. of Meterology, T. F. Malone (Ed.) p. 13. Amer. Meterol. Soc., Boston, Mass. 1951.
5. Johnson, S. P. & Hall, W. C.: Vegetative and fruiting responses of tomatoes to high temperature and light intensity. — Bot. Gaz. 114: 449. 1953.
6. — — Further investigations into the effect of high temperatures and high light intensities on vegetative and fruiting responses of tomatoes. — Bot. Gaz. (In Press.) 1956.
7. Johnson, S. P., Hall, W. C. & Liverman, J. L.: Unpublished experiments. 1956.
8. Laude, H. M.: The nature of summer dormancy in perennial grasses. — Bot. Gaz. 114: 284. 1953.
9. Liverman, J. L. & Bonner J.: The interaction of auxin and light in the growth responses of plants. — Proc. Nat. Acad. Sci. 39: 905. 1953.
10. Liverman J. L., Johnson, M. P., & Starr, L. A.: A reversible photoreaction controlling the expansion of etiolated bean leaves. — Science 121: 440. 1955.
11. Parker, M. W., Hendricks, S. B., Borthwick, H. A. & Scully, N. J.: Action spectrum for the photoperiodic control of floral initiation in short-day plants. — Bot. Gaz. 108: 1. 1946.
12. — — —, Went, F. W.: Spectral sensitivities for leaf and stem growth and of etiolated pea seedlings and their similarity to action spectra for photoperiodism. — Amer. J. Bot. 36: 194. 1949.
13. Swanson, C. P., Rupert, C. P. & Yost, H. T.: Infrared absorption and temperature studies on the buds and chromosomes of Tradescantia paludosa. — Amer. J. Bot. 40: 557. 1953.
14. Thiman, K. V.: Studies on the growth hormone of plants. VI. The distribution of the growth substance in plant tissues. — J. Gen. Physiol. 18: 23. 1934.

Experiments on Development in *Fagus silvatica* by Means of Herbaceous Grafting

By

M. Schaffalitzky de Muckadell

The Arboretum, Hørsholm, Denmark
(Received April 13, 1956)

In a previous paper (1954) existence was proved of a well established juvenile stage in the development of *Fagus silvatica*. This stage is characterized by an ability of the shoots to retain their withered leaves during winter. By this ability the juvenile stage may be distinguished fairly well from more advanced stages in the life cycle of the beech. Such advanced stages may be observed in upper parts of the trees whose shoots shed their leaves in autumn.

It was observed, however, that leaf-retaining power was unevenly distributed throughout the shoots, both in those developing on juvenile plants and in those on juvenile branches of older trees. Generally some of the most apically located leaves were shed at the same time as leaves from developmentally advanced shoots (figure 1). This phenomenon, leaf-shedding in shoot tips, is somewhat similar to that which takes place during transition from the juvenile to the following stage. Also here leaf-shedding in shoot tips is observed. The difference between the phenomena is first noticeable, however, when a new set of shoots has appeared.

Leaf-shedding tips of shoots just entering the following stage produce only new shoots with no winter leaf-retaining power at all. Apically located buds and buds forming in the axil of leaves, which on juvenile shoots are shed in autumn, produce only shoots able to retain most of the leaves in winter.

These observed facts may be explained in two different ways: 1) Transition from juvenile to the following stage begins early in the life of the plant and is manifest in the shoot tips. Transition processes are then inhibited in some

Figure 1. *Five year old beech in the juvenile leaf-retaining stage.* Phot. Jan. 6, 1953. Observe the leaf-shedding shoot tips.

way or other, possibly by hormone action from older parts of the plant. This inhibition is gradually overcome and transition finally becomes complete. 2) Transition is first manifest in meristems and buds which later develop shoots without leaf-retaining power. This agrees with the fact that the leaf-retaining character is not absolute. Under certain conditions — for instance in dense shade — juvenile beeches shed their leaves (Schaffalitzky, 1954).

1. Experiments. From a number of 5—6 year old beeches clearly showing the juvenile characteristics, leaf-shedding tips were cut off in the winter of 1952/53 and used as scions in the spring of 1953.

With the ordinary grafting methods it was not possible to use the extremely slender shoot tips as woody scions, so the basal parts were used. The rootstocks were seedlings in the nursery of the Arboretum and the grafts were transplanted in 1953 and 1955. In December 1955 the 12 existing grafts were big enough to ensure that they were all leaf-retaining.

As the basal parts of the shoot used for scions in this experiment were a very close continuance of the leaf-retaining twigs they might have been more influenced towards juvenility than the very shoot tips.

In another experiment, however, a special herbaceous grafting technique

Table 1. *Results of herbaceous grafting in trees.*

| Date | Species | Number of grafts | |
		initiated	alive following spring
30/5-52	Fagus silvatica	5	4
5/6-53	» »	65	21
15/6-54	Acer pseudoplatanus	10	8
9/6-55	» »	20	15

made possible a transplantation of scions close to the very tips. This method is described below. The grafting was done in June 1953 on branches of an old beech. In this way it was possible to test whether this leafless root stock would influence the grafts towards leaf-shedding. This was not the case, as in December, 1955, all six remaining grafts were found to have retained their leaves.

These two experiments clearly show that it was impossible by grafting to maintain in the shoot tips the ability of shedding their leaves. On the contrary it was proved that such tips were juvenile, although the general and most obvious character of the juvenile stage was lacking. The second explanation is therefore correct.

2. Grafting technique. Herbaceous grafting methods are extensively used for plants with semi-succulent stems, but are generally not recommended for shrubs and trees, although these methods are known to be technically possible (M. G. Kains and L. M. McQuesten, 1947). The main reason for this is probably that normal methods using mature wood, generally give good results. On a large scale herbaceous grafting of Pinus nigra on Pinus silvestris was made more than a hundred years ago in the Forêt de Fontainebleau (Delamarre, 1831; Marrier de Boisdhyver, 1843). Some magnificent specimens produced in this way are still in existence.

The herbaceous grafting method used in the above experiment will be described as it has certain advantages in special cases. It has been used on a small scale in the Arboretum since May 1952. The results obtained are shown in Table 1.

The herbaceous, but not too soft, scion-material is collected immediately before use and kept moist. Simple cleft grafting is made and the graft tied with raffia without wax. The leaves of the scion and of the stock shoot are cut to about one quarter of their size or less. A heavy strip of waterproof cotton is fastened around the shoot just below the bottom of the cleft and a test-tube — length 10 cm, diameter 3 cm — is pulled over the scion and kept in place by the cotton. In this way a high degree of humidity is secured
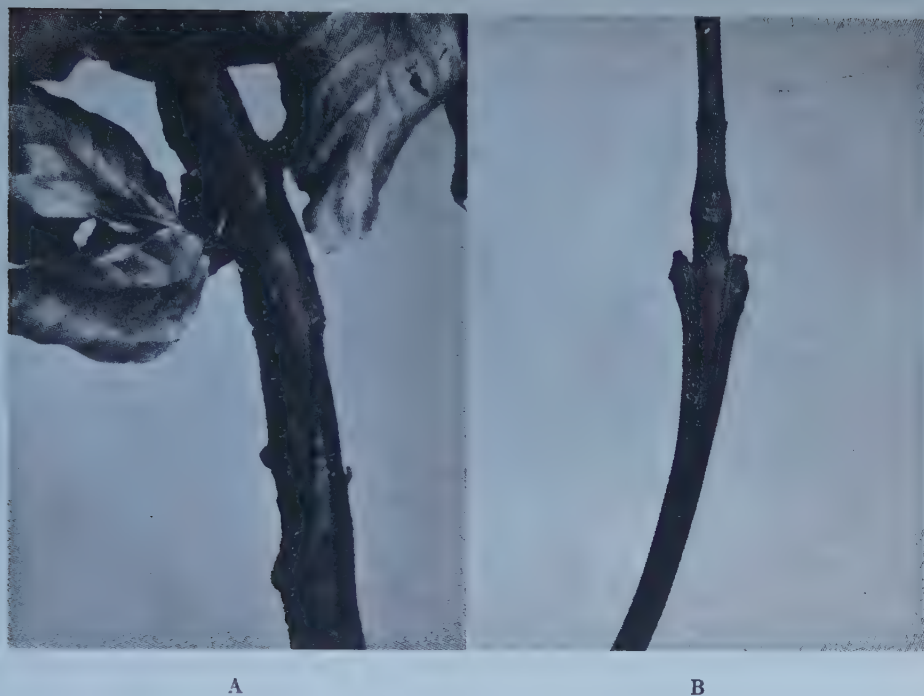
Figure 2. *Herbaceous grafting.* Phot. Feb. 20, 1956. Observe the complete union formed. A: Fagus silvatica. Scion from leafless shoot tip of young leaf-retaining plant. Grafted June 5, 1953, in the crown of an old tree. B: Acer pseudoplatanus. Scion from the top of an old tree. Grafted June 9, 1955 on a stump sprout.

as in a propagating frame. Finally a paper bag is tied round the shoot to provide some shade.

If the stock-shoot tends to droop, because of the heavy tube, it must be tied up. The rather poor results in 1953 were largely the results of not doing so. The terminal bud of the scion at times dipped in water which accumulated in the tube. Lighter material than glass — for example plastic or cellophane — will prevent this contingency. After about three weeks the tubes are removed, but the bags are kept for some time to avoid too strong evaporation.

The main advantages are the extremely quick healing and the complete union formed (figure 2). Furthermore it is possible to graft during the growing season. With Acer pseudoplatanus, spring graftings with mature wood in 1953 and 1954 were complete failures, but herbaceous summer grafting without loss of growing season made experimental work with this species possible.

Summary

Experiments showed that it was impossible by grafting 'to maintain in leafless shoot-tips of juvenile beeches the ability of leaf-shedding. A herbaceous grafting method was used and its importance in certain cases discussed.

References

Delamarre, L. G.: Traité pratique de la culture des pins, 3. éd. — Paris 1831.

Kains, M. G. & L. M. McQuesten: Propagation of plants. — New York 1947.

Marrier de Boisdhyver: Greffage des pins dans la Forêt de Fontainebleau. — Annales Forestières. 2: 220. 1843.

Schaffalitzky de Muckadell, M.: Juvenile stages in woody plants. — Physiol. Plant. 7: 782. 1954.

Uptake of Magnesium by Apple Leaves

By

Kristian Oland and Trygve B. Opland [1]

Ullensvang Research Station, Lofthus, Hardanger, Norway
(Received April 4, 1956)

Introduction

Application of magnesium to plants as foliar sprays has been extensively used as a means to prevent development of symptoms of magnesium deficiency Boynton (2) states that three to five sprays early in the growing season with 2 per cent Epsom salts ($MgSO_4 \cdot 7H_2O$) may prevent the development of deficiency symptoms in apple trees, while the effect of application to the soil is usually delayed by one to three years. According to a recent report by Roach (11) three sprays with 2 per cent magnesium sulphate showed only a transitory effect, whereas nine sprays prevented the symptoms entirely. It is thus obvious that the aerial parts of apple trees are able to absorb magnesium in quantities sufficient to prevent the appearance of deficiency symptoms. The recommended dosages, however, do seem heavy when compared to the amount of the element probably needed for this purpose. This may suggest that the process of uptake is not an effective one.

There seems to exist little knowledge concerning the mechanism of uptake of nutrient elements through leaves. As pointed out by Crafts (6), it is not likely that aqueous solutions enter the leaves to any extent through the stomata. Breazeale *et al.* (4) have shown that tomato plants under conditions of high atmosphere humidity and dry soil, absorb large amounts of water through their aerial parts. For plants under natural conditions, Arvidsson (1) gives examples of leaf absorption of 25—30 per cent of the fallen dew. She

---

[1] The authors' present adresses, Botanical institute, Vollebekk, and Parkveien 61, Bodø, respectively.

fóund that the fresh weight of the leaves increased by 1—2 per cent even if the dew did not remain for more than three to four hours. Roberts *et al.* (12) showed pectinaceous substances to be present in the walls of the epidermal cells of apple leaves, and the outer walls to have intermittent parallel layers of these substances interspersed with cutinized areas. They maintain that the amount and location of pectinaceous substances account for the entrance of water soluble materials.

Although the uptake of nutrient elements through the leaves may be an absorption of aqueous solutions, it was realized that a process by ion exchange, as suggested by Reichenberg and Sutcliffe (10) for plants in general, should be considered as another alternative.

This was in brief the background for the present work. It was decided to study the uptake of magnesium over relatively short periods of time, by analyses of sprayed leaves.

Material and methods

The plants used were maidens of the Norwegian apple variety Torstein, grown partly in nursery and partly in temporary greenhouses when it was necessary to avoid rainfall on the plants.

For investigations on the influence of soil water conditions, the plants were grown in clay pots, the inside walls of which were painted with a bitumen lacquer to prevent loss of water through the pot walls. The soil used was taken from a field where symptoms of magnesium deficiency had previously been found in apple trees and oats. The wilting point and water-holding capacity of the soil were determined according to methods described by Piper (9) and found to be 7.8 and 41.5 per cent respectively, on an oven-dry basis (105° C.). Plants were kept at three soil moisture levels: Series A near the wilting point (7.8—13 per cent, as determined by weighing), series B at an intermediate water content (20—30 per cent), and series C near the waterholding capacity (35—40 per cent water content). The plants were partly kept at these moisture levels throughout the growing season, and partly adjusted to these levels as near up to the time of the actual experiment as possible.

Each single leaf used in the experiments was sprayed separately with a hand-atomiser. Both the upper and the lower surfaces were sprayed. Usually a 5 per cent magnesium sulphate ($MgSO_4 \cdot 7H_2O$) solution was used, and a wetting agent, "Triton", added.

Leaves situated at the same distance either from the top or the base of the shoots, were chosen for each experiment. If an experiment had four treatments, four neighbouring leaves, from each of four plants were chosen and numbered in the same sequence for every replication. A sample was then composed of one leaf from each plant, each leaf having a different number. An exception from this pattern had to be made in an experiment on the redistribution of the absorbed magnesium, in which the leaves from a whole plant had to be used in one sample.

Before analysis the leaves were wiped with a dot of cotton wool repeatedly wetted, and rinsed in distilled water. This washing was found necessary. Mann and

Wallace (7) extracted up to 55 per cent of the total potassium in apple leaves by leaving them submerged in water for seven days, but relatively large amounts were also extracted by soaking the leaves for a short time. Nicholas (8) did not wash tomato leaves sprayed with magnesium sulphate before analysis, Boynton *et al.* (3), working with apple leaves, depended on the washing effect of heavy rain. By the washing used in these experiments, the quantities of calcium and potassium found in the washing water equaled approximately 2 per cent of the leaf content, while corresponding values for magnesium were 7—12 per cent. This higher percentage for the latter element is rather due to a lower content of magnesium in the leaves, than to a higher absolute content in the washing water.

The leaves were dried at 75° C. to a constant weight. Samples for chemical analysis were digested with a 1 : 2 mixture of concentrated nitric acid and perchloric acid. Approximately 1.5 ml of this reagent was needed to digest 100 mg of leaf material. The digestion was carried out at a temperature of about 60° C. until all particles of leaf material had disappeared, and then by increased heating until the digest had become colourless. As perchloric acid in the presence of organic matter is explosive at concentrations exceeding 70 per cent, the digest remained under watchglass cover till colourless. Then the acids were allowed to evaporate at low heat. The residue was dissolved in a few drops of dilute hydrochloric acid and hot water, and transferred to measuring flasks.

Magnesium and calcium were determined by titration with sodium-ethylene-diaminetetra-acetat (Versene) according to Cheng and Bray (5). The apple leaves did not contain interfering metals in concentrations high enough to make removal necessary. This was concluded from available data on the amounts likely to occur, and also from the fact that purification procedures failed to alter the titration results of the original residue solutions. Potassium was determined with a Perkin Elmer Flame Photometer Model A, lithium being used as internal standard.

The statistical treatments of the data are according to Snedecor (14).

Results

Validity of the present approach to the problem of uptake. The pre-requisites for studying the uptake by analysis of sprayed leaves would be that no significant amount of the absorbed magnesium should be carried away to other plant parts during the experimental period, and further, that it should be possible to apply quantities of magnesium which would allow a relatively large uptake without causing visual damage to the leaves.

It was found that spraying with concentrations up to 5 per cent (w/v) of magnesium sulphate in no case resulted in any visual damage to the leaves, whereas higher concentrations might cause some scorching one to two days after application. As will be seen from Figure 1, spraying with 1 per cent solution gave no significant increase in leaf magnesium, whereas a 5-per cent solution gave highly significant increase. Further, the amount of magnesium remaining on the leaf surfaces was proportional to the concentration in the
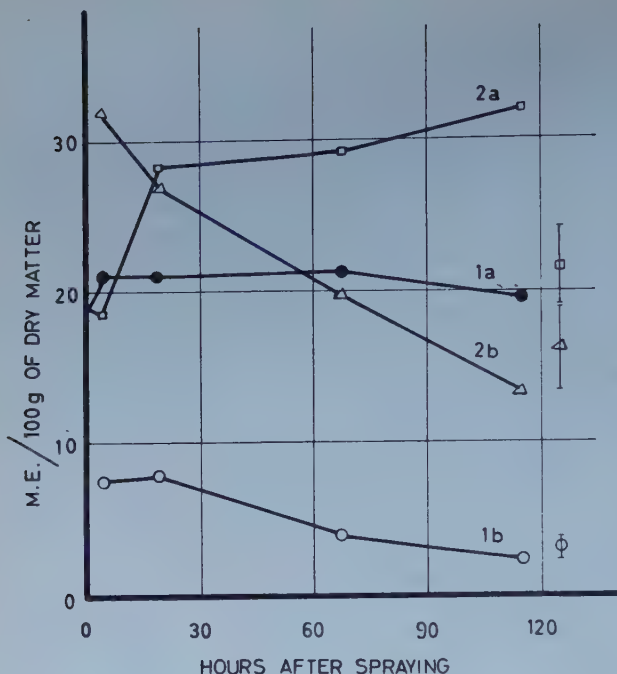
27

Figure 1. *The magnesium content of young leaves and surface magnesium after spraying with 1 and 5 per cent aqueous solutions of magnesium sulphate.* Time of spraying 3 P.M. *1 a*, Mg content; *1 b*, surface Mg after application of 1 per cent solution; *2 a*, Mg content; *2 b*, surface Mg after application of 5 per cent solution. The least significant differences, P = 0.05, (L.s.d.) are graphed at the right hand side of the figure.

solution applied, in this experiment $1/3$ and $1 1/2$ times the amount initially found in the leaves for the 1 and 5-per cent solutions respectively.

Figure 2 shows the result of an experiment where also the "shoot" carrying the leaves and the undeveloped leaves, "the top", above the sprayed ones. were analysed. At the time of spraying the plants had only reached a height of 14 cm above the place of budding, and they grew 6 cm during the four days the experiment lasted. Quite a rapid change in the magnesium content took place during the experiment, and might be due to plant development. This had to be allowed for in the interpretation of the results, and an analysis of covariance has therefore been used for the magnesium content in "shoot" and "top", which have been adjusted to length and dry weight respectively. An adjustment of the data on leaf content and surface magnesium has also been made before the analyses of variance because of a small increase in the area of the sprayed leaves. While there is a highly significant uptake of magnesium according to the analyses of both leaves and washing water, there is no significant change in the content of other plant parts due to spraying. The changes in the magnesium content in "shoot" and "top" during the experimental period is fairly well accounted for as a result of the plant development.
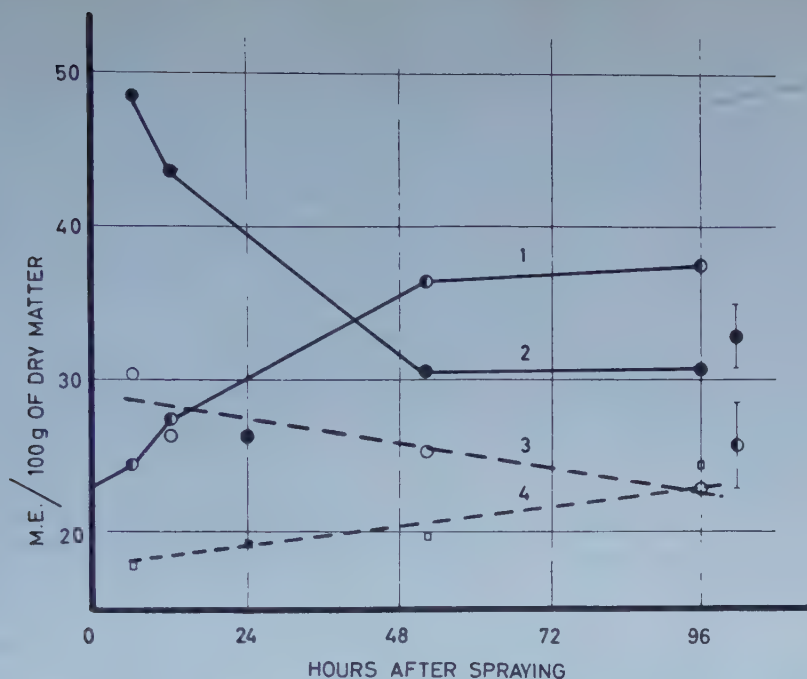
Figure 2. *Experiment on the redistribution of absorbed magnesium.* The leaves were sprayed at 9 A.M. with a 5 per cent magnesium sulphate solution. *1,* magnesium content of the sprayed leaves; *2,* leaf surface magnesium; *3,* expected trend in the magnesium content of the "shoot" because of increasing length. The open rings show the original analytical results obtained after spraying, the filled one represents unsprayed plants. *4,* the expected trend in the magnesium content of the unsprayd "top" due to increasing dry weight. The open squares show the original analytical results obtained after spraying, the filled square represents unsprayed plants. L.s.d.'s at the right hand side of the figure.

In the two experiments reported up to now, it is possible to calculate the uptake from the analyses of the leaves, as well as those of the washing water. An analysis of variance shows that there is no significant difference in the uptake, as calculated in these two ways. There is a somewhat lower variability in the analyses of the surface magnesium than in the leaf analyses. Even so, it may be considered safer to use analyses of leaves in experiments on uptake, as a 5 per cent spray leaves salt crystals on the surface which may fall off if the leaves are shaken.

From these experiments it may be concluded that spraying with 5 per cent magnesium sulphate solution in uptake studies seems satisfactory. There is no indication that the magnesium absorbed by the sprayed leaves is re-distributed to other plant parts during experiments lasting a few days.

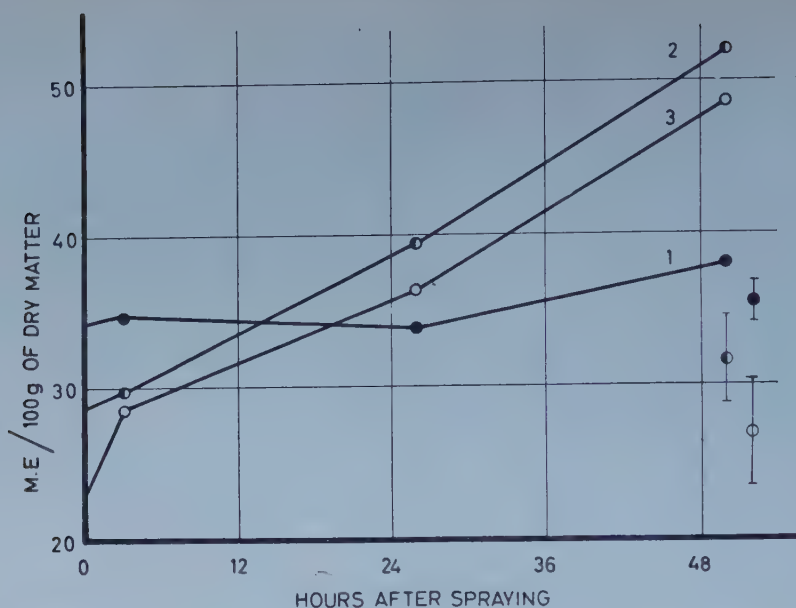*Influence on uptake of soil water conditions and rewetting of sprayed*

Figure 3. *Magnesium content of sprayed, young leaves of plants grown at different soil moisture levels.* Application of spray at noon. *1*, plants in dry soil; *2*, plants in medium wet soil; and *3*, plants in wet soil. L.s.d.'s at the right hand side of the figure.

leaves. Figure 3 shows the uptake by young leaves at three soil moisture levels. The sprayed leaves were the latest fully developed leaves from the top of the shoots. The plants had been kept at the chosen moisture levels throughout the growing season. This had resulted in mean leaf areas of 13.4, 32.0, and 38.1 sq.cm for plants in dry, medium wet, and wet soils respectively. There is a significant uptake at all moisture levels, but the plants in dry soil show a lower uptake than the medium wet and wet series.

Figure 4 gives the results of the same type of experiment repeated on older leaves from the basal end of the shoots, and these shoots were at the point of forming end buds at the time of the experiment. The plants were adjusted to the three moisture levels as near up to the time of the experiment as possible (a period of 16 days). In this experiment a significant uptake by the leaves was found only in the plants kept at the highest soil moisture level, and the increase in leaf magnesium is slight, even for this group.

Sprayed leaves at all moisture levels were rewetted by applying distilled water with a hand atomiser, but this treatment did not result in increased uptake.

The conclusions of these experiments would be that conditions that were likely to lead to increased water absorption by the leaves, showed no
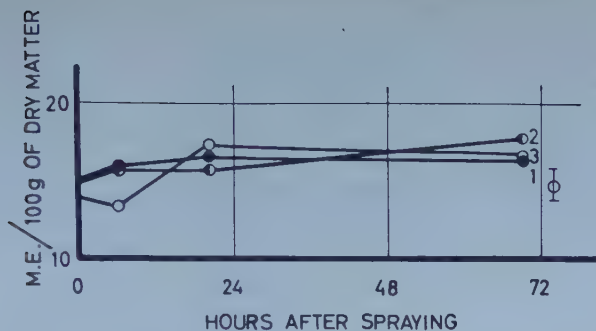
Figure 4. *Magnesium content of sprayed, relatively old leaves of plants grown at different soil moisture levels.* The leaves were sprayed at 4 P.M. *1*, plants in dry soil; *2*, plants in medium wet soil; and *3*, plants in wet soil. L.s.d.'s at the right hand side of the figure.

connection with the magnesium uptake. The leaf age is indicated as an important factor in the magnesium uptake. The explanation of the poor absorption by plants in dry soil may be that under such conditions the leaves become "physiologically aged" relatively early.

Spraying at different hours during the day. If the leaves absorb magnesium by ion exchange, the diurnal shift in acid production may influence the uptake.

Table 1 gives the results of an experiment where relatively old leaves were sprayed, either in the early afternoon, or in the evening, and sampled for analysis after 23 hours. There was no significant absorption by these leaves when magnesium was applied during the day, but the evening spraying lead to a very large and highly significant uptake.

The uptake by young and old leaves, belonging to the same plants, was compared after spraying in the evening, and the results are given in Figure 5. The old leaves absorbed a large amount the first night after the application and did not absorb significantly later on. The young leaves represent a somewhat different picture, as these did absorb significant quantities after the first night.

These experiments show that relatively old apple leaves may absorb large

Table 1. *The effect of magnesium sulphate solution applied at different hours during the day.* The sprayed leaves were fully developed approximately two months before the experiment. There was no working light after spraying at 9 o'clock.

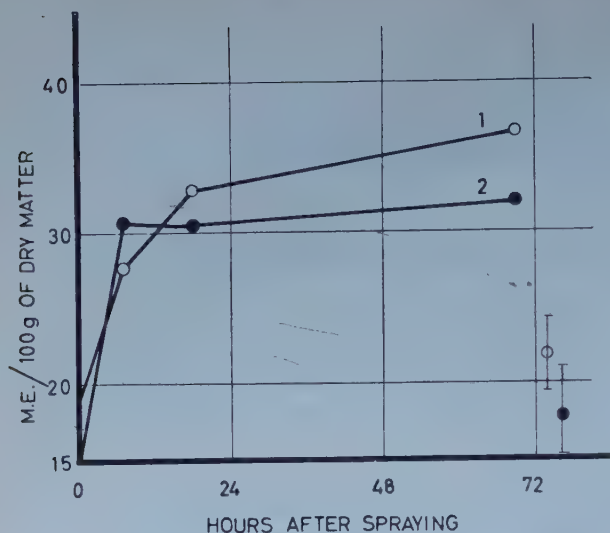| Time of spraying	Control	2.30 P. M.	9.00 P. M.	L. s. d.
Absorption time, hours	0	23	23	
Mg content, m.e./100 g dry matter ...	11.1	13.2	24.3	4.38

Figure 5. *A comparison of the absorption by young and old leaves of the same plants after spraying in the evening, just before darkness (9.30 P.M.) 1, the latest fully developed leaves; 2, approximately two-month-old leaves. L.s.d.'s at the right hand side of the figure.*

quantities of magnesium if spraying is carried out during the evening. It is indicated that the hour of application is a dominant factor for rapid magnesium absorption by young leaves as well.

Some preliminary experiments on application to old leaves during the day of solutions of the magnesium salts of acetic-, citric-, and succinic-acid, showed a strikingly high absorption from the magnesium acetate when compared to the sulphate. The magnesium content of the leaves was quickly tripled on the application of the acetate. However, a concentration equivalent to 5 per cent magnesium sulphate caused severe injury to the leaves. It was found that a concentration equivalent to 1 per cent gave no visual damage to the leaves, but experiments on uptake have not been carried out with this solution.

Changes in potassium and calcium content in leaves sprayed with magnesium. The samples from all experiments have been analysed for calcium and to a large extent for potassium also.

No striking or significant change in the potassium content of the leaves have been observed in these experiments. This applies in most cases to calcium as well. In the experiments reported in Figures 1 and 2, however, some peculiar changes in the calcium content of the sprayed leaves were observed. The results of the calcium analyses are given in Figure 6. A few hours (4 and 6) after spraying a sudden drop was found in the calcium content of the leaves sprayed with 5 per cent magnesium sulphate. This concentration resulted in a large uptake. No corresponding drop in the

Figure 6. *Changes in the calcium content of the leaves sprayed with magnesium sulphate. 1,* leaves sprayed with a 5 per cent solution (corresponds to *1 a* of Figure 2); *2,* leaves sprayed with a 5 per cent solution (corresponds to *2 a* of Figure 1); and *3,* leaves sprayed with a 1 per cent solution (corresponds to *1 a* of Figure 1). L.s.d.'s at the right hand side of the figure.
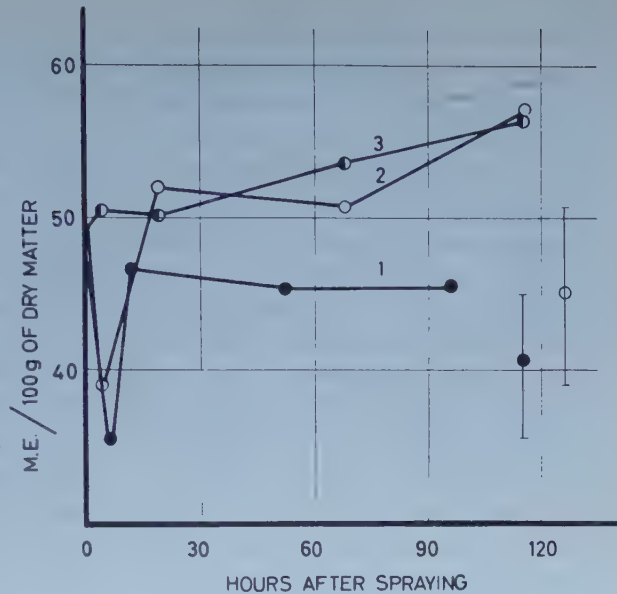
calcium content was found for leaves sprayed with 1 per cent magnesium sulphate.

At least part of the calcium lost by the leaves at this point was found in the washing water. And even after the leaves had regained calcium to a prespray level, a high content was found on the leaf surface. The actual figures are not presented as they may be somewhat low due to slow titration of the calcium in gypsum, which possibly was formed on the leaf surface.

It seems likely that the sampling time, shortly after spraying, but before any significant amount of magnesium had been absorbed, was the critical one for the detection of this drop in the calcium content of the leaves.

Discussion

It is apparent that the uptake of magnesium by apple leaves can be studied easily by analyses of sprayed leaves, as large quantities of magnesium sulphate can be applied without any visual injury resulting, and the absorbed magnesium does not appear to be redistributed to other plant parts in the following few days.

Although the experiments reported cannot be accepted as a final proof that magnesium does not enter the leaves in aqueous solution, they would serve as an indication that this way of entrance is not an important one. The

leaf age is of importance for the entrance of magnesium into the leaves, but the by far most decisive factor seems to be the hour of spraying. The large uptake after spraying in the evening points to ion exchange as the mechanism involved in the absorption. Internal release of hydrogen ions for exchange would be most likely to occur after the probable shift in the production of organic acids during the evening. The diurnal fluctuation in the organic acid content is best known in the metabolism of succulents. According to the conclusions by Schwarze (13), however, this fluctuation is not found in succulents only, but may be of common occurrence in plants. Thomas *et al.* (15) states that increase in titrable acidity during the night is not an invariable attribute of succulence. The diurnal fluctuation in acid metabolism is most pronounced in old leaves, while the changes in the organic acid content of young leaves seem to be independent of day and night. It seems thus most satisfactory to accept ion exchange as the absorbing mechanism.

The loss of calcium at the beginning of the absorption may imply that the ion exchange is carried on along chains of pectic acid. The calcium salt of this acid is most likely to occur before magnesium is applied, and thus the drop in calcium content would be due only to the expected release of cations by the exchanger. This assumption is not fully satisfactory, however, because the prespray calcium content seems to be regained while the absorption of magnesium is still going on.

As to the use of magnesium nutrition through the leaves, these experiments should serve to indicate that in practice some changes would be profitable. At present it is not clear how long in advance of darkness the spraying can be performed with the same large uptake, which has been shown to occur in these experiments. The length of this period may be somewhat dependent on the climatic conditions, so that in places where the spray does not dry very quickly the period may afford ample time for application over more extensive areas. If it should appear that spraying during the day represents the most desirable practice, it may be worth while to study if and how acetic acid can be used as a means to increase the absorption.

Summary

In the present investigation it was found that apple leaves can be sprayed with a 5 per cent magnesium sulphate solution with no visual damage resulting. No indication was found that the magnesium absorbed by the leaves is carried away to other plant parts. The absorption seems to be little influenced by soil water conditions, and rewetting of the sprayed leaves did not increase the uptake. Young leaves absorb magnesium readily, while old

leaves absorb very little or nothing at all, if sprayed during the day. When sprayed in the evening, just before darkness, old leaves absorb large amounts of magnesium. The hour of spraying seems also to be of importance for rapid absorption by young leaves. Preliminary experiments have shown that large amounts of magnesium are absorbed if the element is applied during the day as solution of the salt of acetic acid, but leaf damage may limit the value of this treatment. In two of the experiments reported, a sudden drop in the calcium content of the leaves was detected. It occurred shortly after spraying, but before any significant increase in magnesium content was found. At least part of the calcium lost by the leaves was refound on the leaf surface.

References

1. Arvidsson, Inga: Austrocknungs- und Dürreresistenzenverhältnisse einiger Repräsentanten Ölandischer Pflanzenvereine nebst Bemerkungen über Wasserabsorption durch oberirdische Organe. — Oikos, Supplementum 1: 1. 1951.
2. Boynton, D.: Nutrition by foliar application. — Ann. rev. plant physiol. 5: 31. 1954.
3. Boynton, D., Cain, J. C. & Van Geluwe, J.: Incipient magnesium deficiency in some New York apple orchards. — Proc. am. soc. hort. sci. 42: 95. 1943.
4. Breazeale, E. L., McGeorge, W. T. & Breazeale, J. F.: Moisture absorption by plants from an atmosphere of high humidity. — Plant physiol. 25: 413. 1950.
5. Cheng, K. L. & Bray, R. H.: Determination of calcium and magnesium in soil and plant material. — Soil sci. 72: 449. 1951.
6. Crafts, A. S.: Sulfuric acid as a penetrating agent in arsenical sprays for weed control. — Hilgardia 8: 125. 1933.
7. Mann, C. E. T. & Wallace, T.: The effects of the leaching with cold water on the foliage of the apple. — Journ. pom. hort. sci. 4: 147. 1925.
8. Nicholas, D. J. D.: Experiments on correcting magnesium deficiency in glasshouse tomatoes. — Journ. pom. hort. sci. 24: 1. 1948.
9. Piper, C. S.: Soil and plant analysis. — New York 1944.
10. Reichenberg, D. & Sutcliffe, J. F.: Absorption of ions by plants. — Nature 174: 1047. 1954.
11. Roach, W. A.: Studies in nutrition. — Ann. rep. East Malling res. sta. 1954: 32.
12. Roberts, E. A., Southwick, M. D. & Palmiter, D. H.: A michrochemical examination of McIntosh apple leaves showing relationship of cell wall constituents to penetration of spray solutions. — Plant physiol. 23: 557. 1948.
13. Schwarze, P.: Ein Beitrag zur Kenntnis des Säurestoffwechsels nichtsukkulenter Pflanzen. — Planta 18: 168. 1933.
14. Snedecor, G. W.: Statistical methods. — Iowa. 1948.
15. Thomas, M., Ranson, S. L. & Richardson, J. A.: Plant physiology. — London 1956.

The Gas Exchange of Flax Seeds in Relation to Temperature

II. Experiments with Germinating Seeds

By

HENRIK HALVORSEN

The Botanical Laboratory of Oslo University
(Received: April 7, 1956)

Introduction

An earlier report (Halvorsen, 1955) has dealt with the gas exchange of immature flax seeds in relation to temperature. The present paper concerns the respiration of germinating flax seeds under different temperature conditions.

Previous works on the gas exchange of germinating fatty seeds are numerous. In flax seeds as in other fat-storing seeds, characteristic changes of the respiratory quotient have been observed (Bonnier and Mangin, 1884, Stiles and Leach, 1933). In general it appears that during germination of fat-containing seeds, the RQ is about one during a short initial period. The quotient then decreases to a minimum value and, during a final period, the RQ rises and may approach unity.

As to the absolute RQ-values of germinating flax seeds there seems to be little agreement. The theoretical RQ-value for the oxidation of fats is about 0.7, but much lower values have been recorded in experiments. Bonnier and Mangin (1884) determined the respiratory quotient of flax during the first 17 days of germination. A minimum value of about 0.35 was observed after 4 days of germination. Murlin (1933), during his study of the respiratory metabolism in the germinating castor bean, also made records of the RQ of flax seeds at early stages of germination. The RQ varied from 0.487 to 0.750, the average being 0.633.

It has been suggested that the low quotients indicate the conversion of fats

to carbohydrates. This process, which is an oxidation without evolution of carbon dioxide, probably proceeds simultaneously with and more rapidly than the oxidation of sugars to carbon dioxide and water and may thus account for lower RQ-values than the theoretical one.

Studies of seed respiration at different temperatures have usually been restricted to measurements of respiration intensity, the relation between temperature and RQ having received little attention. In a short paper, Lang (1933) reported that the RQ of germinating seeds of *Lupinus albus* was a function of temperature, and that great variations in the RQ occurred at different temperatures.

The effect of temperature on the RQ is related to the various chemical processes which take place in the seeds. During germination of fatty seeds, fats are converted to carbohydrates which accumulate in the seeds. There is also an increase in the content of free acid and in the proportion of saturated fatty acids. The iodine number decreases steadily during germination.

Both the saturation and breakdown of fats are accompanied by gas exchange, the extent of which is dependent upon many different chemical processes. Consequently, the significance of the respiratory quotient is somewhat doubtful. However, if the temperature exerts a qualitative effect upon fat metabolism, it should appear in the metabolic gas exchange. A determination of both oxygen consumption and carbon dioxide evolution at different temperatures would therefore be valuable in the study of fat metabolism and its relationship to temperature.

Material and method

The seeds of *Linum usitatissimum* used in the present study were of an oil variety, Valuta, from the Swedish Seed Association (Svenska Utsädesföreningen), Svalöf. The seeds had previously been treated with a disinfectant (Panogen), and before germination they were washed for a few minutes in distilled water. The seeds were then placed direct on moist filter paper in Petri dishes for germination at constant temperatures in the dark. The disinfectant was not completely removed in the distilled water, but the chemical did not seem to have any retarding effect upon the germination of the seeds. The microbial activity had apparently been strongly inhibited. Although visible development of microorganisms was rare, it is still possible that a small part of the respiratory activity may be attributed to micro-organisms in the mucous coat of the flax seeds.

In preliminary tests it was observed that submergance in tap water for 8, 12, or 24 hours at 20° C had an unfavourable effect on the germination and growth of the seedlings. Consequently presoaking was not applied in the later experiments.

Seeds were placed to germinate at 30°, 25°, 20°, 15°, 10°, and 5° C., and samples were removed daily for determining the respiration. Temperature control was satis-

factorily obtained in automatically controlled water baths. For measuring the gas exchange, modified Warburg respirometers were used. Both the temperature control system and the Warburg respirometers have been described in an earlier report (Halvorsen, 1955).

Depending on the stage of germination, samples containing 5—10 seeds or seedlings were placed in the respirometers. 4 parallel samples were run and the period of exposure in the respirometers was usually 5—6 hours, except in some cases where it was extended to 10 hours. The rate of carbon dioxide evolution and oxygen absorption was expresed in terms of µl gas per 10 seeds per hour.

Determinations of the gas exchange were made for 7—9 days. However, at 5° C germination was very slow and records of respiratory activity were therefore made after 10, 17 and 25 days of germination.

Results

Both gas exchange and seedling growth were observed in the early stages of germination and development. As can be read from Table 1 the flax seeds germinated very rapidly at the higher temperatures, the hypocotyl tip becoming visible after 15—20 hours. At 5° C the hypocotyl did not emerge until the 10th day, but when the seed coat had been ruptured, seedlings developed even at this temperature. In spite of the rapid initial development at 25° and 30° C. the highest growth intensity occurred at 20° and 15° C., after 5—7 days of germination.

The respiration intensity at different temperatures during the early development of flax seedlings is plotted in Figures 1—2 and in Table 2. The various curves demonstrate what has been called "the grand period of respiration". At 30° and 25° C. the increase in the rate of respiration was

Table 1. *Length of flax hypocotyls during germination at different temperatures.* Seedlings kept in the dark. Lengths given in mm. Average values of 25 seeds.

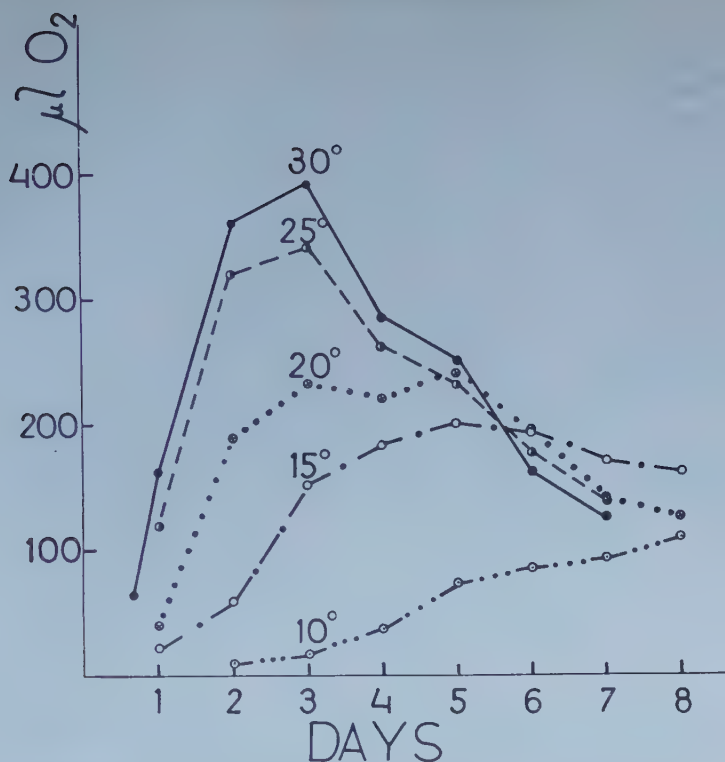| Germination period Days	Temperature °C					
	30	25	20	15	10	5
1	4	3	1			
2	14	17	10	5		
3	22	27	20	10	1	
4	32	39	33	22	3	
5	39	50	44	36	7	
6	45	60	68	58	11	
7	—	—	84	80	16	
8					26	
10						1
17						8
25						20

Figure 1. *Oxygen absorption of flax seeds during germination at different temperatures. Values given as µl per 10 seeds per hour. Average values of 4 parallel samples.*

extremely rapid, maximum values being reached after 3 days of germination. The downward slope of the curves was also much steeper than for the other temperatures.

At 20° and 15° C. maximum respiration rates were reached after about 5 days of germination. Later the rate remained constant or decreased only slowly.

The respiration increase at 10° C. continued for the first 8—9 days of

Table 2. *Respiration rate and RQ-values of germinating flax seeds at 5° C. Carbon dioxide evolution and oxygen absorption given as µl gas per 10 seeds per hour. Average values of 4 parallel samples.*

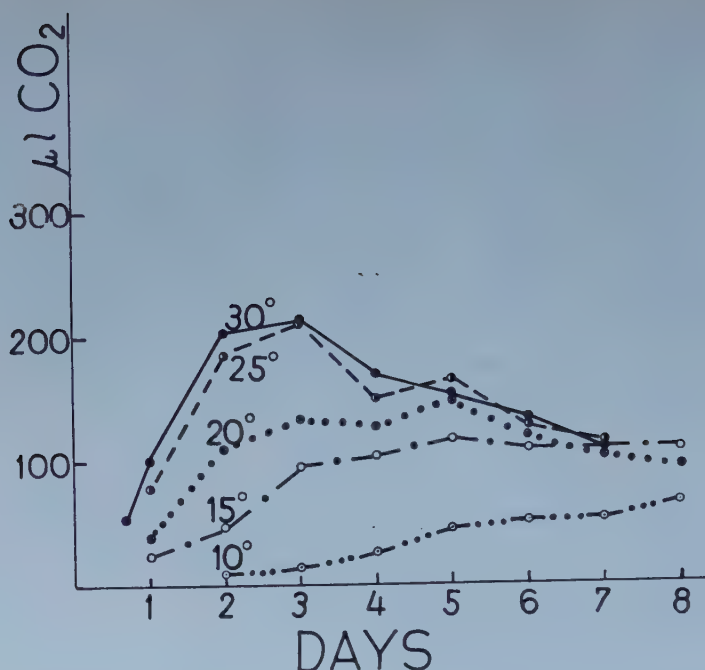| Germination period Days	CO_2	O_2	RQ
10	10.5	14.0	0.74
17	24.5	38.5	0.64
25	20.0	35.0	0.57

Figure 2. *Carbon dioxide evolution of flax seeds during germination at different temperatures.* Values given as µl per 10 seeds per hour. Average values of 4 parallel samples.

germination when a maximum was probably reached. An interesting fact is that on the 6th and 7th day of germination only small differences in respiration rate were found at temperatures between 30° and 15° C. At this time the respiration rate had diminished rapidly at the higher temperatures, thus approaching the more constant respiratory rates at 20° and 15° C.

The changes of the RQ during the development of flax seedlings are plotted in Figure 3. As germination proceeded the RQ-values at all temperatures showed the characteristic fall from about one to minimum values below the theoretical value for the oxidation of fats. Minimum RQ-values were reached at different points of time after germination, depending on the temperature

Table 3. *Minimum RQ-values of flax seedlings at different temperatures.*

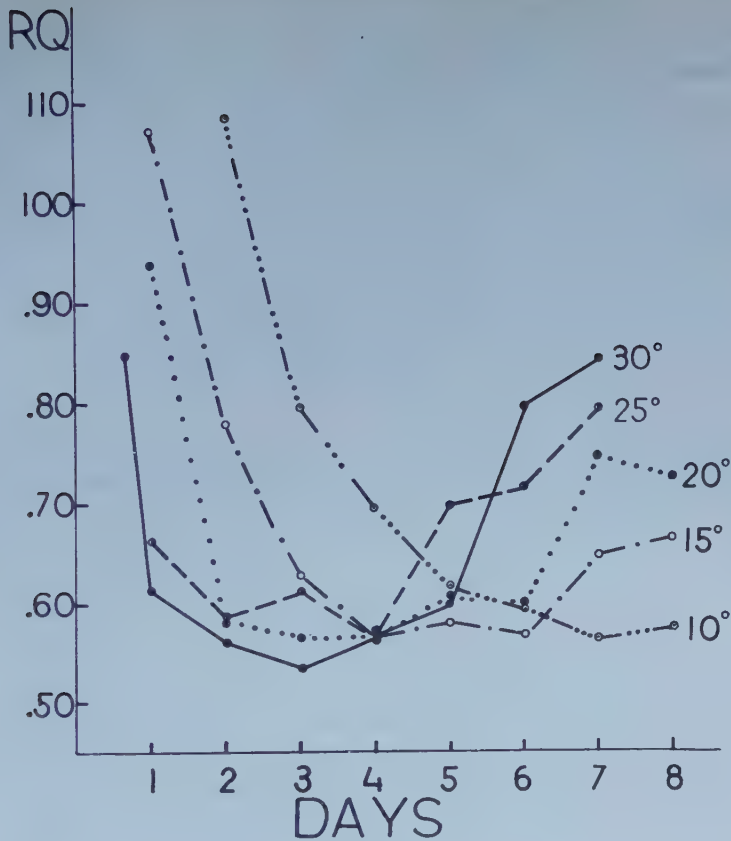| Date	Temperature °C					
	30	25	20	15	10	0
Minimum RQ-values	0.54	0.57	0.57	0.57	0.57	0.57
Age of seedling in days ...	3	4	4	6	7	25

Figure 3. *The respiratory quotient of flax seeds during germination at different temperatures.*

to which the seeds had been exposed. At 30° C. the lowest RQ was recorded on the 3rd day whereas at 10° C. the fall in the quotient continued for 7—8 days. Similarly, the following increase occurred much earlier at high than at low temperatures.

When the corresponding RQ-values and respiration rates are compared it seems that at every temperature level the minimum quotients coincided with maximum intensity.

Further it will be seen from Table 3 that the minimum RQ-values observed at the various temperature levels differed only negligibly. A minimum value of 0.57 was calculated for all the temperatures in consideration, except at 30° C. where the average figure was 0.54. The lowest individual RQ-value 0.48 was observed at 30° C. after 3 days.

In an additional series of experiments, flax seedlings germinated at 15° C.

Table 4. *Respiration rates and RQ-values of 3-day-old flax seedlings at different tempe-ratures.* Seedlings previously germinated at 15° C. CO_2-evolution and O_2-absorption given as µl gas per 10 seeds per hour.

Time in hours	Temperature °C											
	30			20			10			0		
	CO_2	O_2	RQ	CO_2	O_2	RQ	CO_2	O_2	RQ	CO_2	O_2	RQ
2	214	331	0.65	115	197	0.59	46	75	0.61			
4	225	351	0.64	118	203	0.58	46	75	0.61	6.5	10.5	0.62
6	208	340	0.61	122	206	0.59	48	77	0.62	—	—	—
8	205	328	0.63	120	200	0.60	50	80	0.63	7	11	0.63
10	195	315	0.62	121	201	0.60	51	80	0.64	7	11	0.63

for 3 days were tested for respiration during 10 hours at 30°, 20°, 10° and 0° C. The data presented in Table 4 show varying respiration rates, but no significant differences in RQ.

Discussion

The present work confirms some of the earlier work on seed respiration and provides new information about the gas exchange of flax seeds germinating under different temperature conditions.

The germination data indicate that the minimum temperature for germination was about 5° C., possibly one or two centigrades lower, when flax seeds were kept at a constant temperature. No germination was observed at 0° C. during a period of 20 days, nor could any respiratory activity be detected by the method used. However, when transferring seedlings from 15° C. to 0° C. a low respiration intensity could be observed during the next 10 hours. Accordingly it seems that the enzymic activation was too slow to initiate germination when seeds were kept continuously at the freezing point.

The optimum temperature for seed development seemed to be between 15° and 20° C. Although the initial growth was very rapid at 30° and 25° C. the highest growth rates were found at medium temperatures. However, the retardation of growth after 3 or 4 days at the two highest temperatures could, of course be due to exhaustion of reserve fats or to deficiency of minerals under the present conditions of germination.

In accordance with previous results (Stiles and Leach, 1933) the respiration intensity usually increased from a low initial value to a maximum and then gradually fell off. Different temperature conditions significantly influenced the length of time necessary to reach maximum respiration. At 30° C. maximum respiration ocurred after about 3 days of germination, whereas

at 10° C. the increase in respiration rate continued for 7—8 days. It is interesting to note that at all temperature levels the period of maximum respiration corresponded roughly to the period of minimum RQ-values, but showed no definite correlation to the hypocotyl length.

In his study of the germination of lettuce seeds, Griffiths (1938) reported that in seeds germinated at 20° C. the most pronounced lipase activity and fat hydrolysis occurred after about 48 hours. It is reasonable to assume that in the present experiments the most active fat transformation occurred at a period of maximum oxygen absorption when the RQ-values were at minimum. As stated already the time needed to reach this stage depended upon the germination temperature, varying from about 3 days at 30° to about 7 days at 10° C.

In contrast to earlier observations of fatty seeds (Tang, 1932) the RQ seemed to be only slightly affected by temperature. Approximately the same minimum RQ-values were recorded at all temperature levels (Table 3). The minimum values observed in the present study are lower than the theoretical value for complete fat oxidation. Usually low RQ-values are considered to indicate a more rapid conversion of fat to sugar than combustion of sugar to carbon dioxide and water. However, in the present case the difference between the minimum RQ and the theoretical value is not very great, indicating that only small amounts of sugar have accumulated as a result of fat conversion. When transferring 3-day-old seedlings germinated at 15° C. to temperatures from 0° to 30° C. (Table 4), no significant changes in RQ were found during the next 10 hours. This observation strongly supports the conclusion that the temperature mainly influenced the rate of respiration and development. Thus, although the observations do not definitely exclude a qualitative temperature effect upon fat metabolism in germinating flax, it seems more likely that the breakdown of fats follows the same chemical pathways at any temperature within the range investigated.

Summary

1) With modified Warburg respirometers observations were made of the gas exchange of germinating flax seeds at different temperature levels. Oxygen absorption and carbon dioxide evolution were recorded at 1-day intervals during germination and early development of the seedlings.

2) As germination proceeded the rate of respiration increased from a low initial value to a maximum. The length of the period during which the increase took place was markedly influenced by the temperature and varied from 3 days at 30° C. to about 3 weeks at 5° C.

3) From initial values of about 1 the RQ decreased to a minimum value where it again increased gradually. The RQ-curves were also influenced by different temperature conditions, but regardless of temperature about the same minimum RQ-value was recorded. At 30° C. the minimum quotient was found to be 0.54. At 25°, 20°, 15°, 10° and 5° C. a minimum value of 0.57 was observed.

References

1. Bonnier, G. & Mangin, L.: Respiration des tissues sans chlorophylle. — Ann. Sci. Nat. Ser. 6, 18: 293. 1884.
2. Fernandes, D. S.: Aërobe und anäerobe Atmung bei Keimlingen von Pisum sativum. — Rec. Trav. Bot. Neerl. 20: 107. 1923.
3. Griffiths, A. E.: Observations on the germination of lettuce seed. — Contr. Boyce Thompson Inst. 9: 329. 1938.
4. Halvorsen, Henrik: The gas exchange of flax seeds in relation to temperature. I. Experiments with immature seeds and capsules. — Physiol. Plant. 8: 501. 1955.
5. Murlin, J. R.: The conversion of fat to carbohydrate in the germinating castor beans. I. The respiratory metabolism. — Gen. Phys. 17: 283. 1933.
6. Stiles, W. & Leach, W.: Researches on plant respiration. II. Variations in the respiratory quotient during germination. — Proc. Roy. Soc., B. 113: 405. 1933.
7. Tang, P. S.: On the respiratory quotient of Lupinus albus as a function of temperature. Gen. Phys. 15: 561. 1932.

The Effect of Gaseous Ozone, Hexene, and Their Reaction Products upon the Respiration of Lemon Fruit

By

Glenn W. Todd

Department of Plant Biochemistry, University of California
Citrus Experiment Station, Riverside, California
(Received April 16, 1956)

The visible injury to many plants growing in the Los Angeles basin due to air pollutants is now well documented (12, 13, 15, 16). This injury to herbaceous plants can be reproduced in the laboratory by exposing susceptible plants to a mixture of unsaturated hydrocarbons and ozone (6, 16). Visible damage does not occur to some plants after exposure to these air pollutants, although there are indications that physiological changes may take place. Koritz and Went (11) demonstrated that smog can reduce the rate of growth and that the rate of transpiration was decreased by exposure to ozonated 1-hexene. Erickson and Wedding found that photosynthesis is inhibited (4) and changes in permeability occur after plants are exposed to ozonated 1-hexene (18). It is possible that these physiological responses may be observed when susceptible plants are exposed to concentrations of pollutants too low to produce visible symptoms.

Damage to citrus trees from polluted air has not been observed and damage to fruit is not common. There is the possibility, of course, that even though there are no visible symptoms on the trees or fruit there may be an over-all decrease in the growth of the fruit. The change in respiration after exposure of fruit to these gaseous pollutants would be one method of measuring a response since plant respiration responds to many narcotic and toxic substances. This could give some indirect information as to what effects might be expected from fruit exposed on the trees in a polluted atmosphere.

The results of Haagen-Smit *et al.* (6) showed that the 5, 6, and 7 carbon unsaturated olefins reacted with ozone give damage most typical of that occurring in the Los Angeles basin from air pollutants. For the present study 1-hexene was chosen as the olefin. Besides determining the effects of ozonated hexene on fruit respiration, it is of interest to measure the response to hexene and ozone individually. It has been reported (8, 16) that plants are affected by ozone, although no respiration studies were made. Hydrocarbons in the form of oils applied to the leaves have been reported to change the respiration rate (5, 10, 14) although this change appears to be due to the acids and peroxides present in the oil (9).

Materials and Methods

The lemon fruit used were obtained from local packing house sources. They were commercially mature and were selected for their uniformity of size and color. The dark and light green fruit were obtained just after washing, thus receiving very little handling in the packing shed. All of the ripe yellow lemons used had been stored for some time and were waxed. The conditions to which the fruit were exposed and their color is given in each experiment.

Lots containing 30 fruit each were weighed and then placed in 2 1/2 gallon glass containers which were supplied with a carefully controlled air stream metered at the rate of about 30 liters/hour. The room temperature was $20°$ C $\pm 0.5°$ C.

The carbon dioxide released from the fruit was determined by the method of Claypool and Keefer (3). This carbon dioxide was assumed to be due to respiration and was calculated as the amount of CO_2 released in mg./kg. fresh weight/hr. The results given in the figures were calculated as follows:

$$\frac{\text{mg. } CO_2/\text{kg. fresh wt./hr. of treated fruit}}{\text{mg. } CO_2/\text{kg. fresh wt./hr. of control fruit}} = \text{respiration as per cent of control.}$$

The ozone was produced by passing compressed cylinder oxygen through a corona discharge tube after which it was mixed with a stream of hexene gas produced by bubbling nitrogen gas through liquid 1-hexene (obtained from Phillips Petroleum Co.; Technical grade 95 mol % minimum). The rates of flow of the various components were controlled by passing them through small calibrated capillaries. The concentrations used are given in the various figures and are calculated as the final volume concentration as though there were no reactions taking place. In the case of hexene plus ozone there is a reaction and the oxidant that was present was measured either with the ferrous thiocyanate reagent (17) or the phenolphthalin reagent (7). The thiocyanate reagent probably measures primarily peroxides formed in the reaction between ozone and hexene and the phenolphthalin reagent probably measures peroxides and any unreacted ozone in these gasous mixtures.

The first respiration readings were obtained at least an hour after the gas treatment was stopped so that fresh air could replace the contaminated air. The pretreatment respiration rates were determined and the post-treatment respiration rates

have been corrected for any differences between fruit lots at the beginning of the experiment.

The data were statistically analyzed using a t-test. Treatments were compared with controls at each sampling time.

Results

The respiratory response of mature yellow lemon fruit to hexene alone, ozone alone, and the two when present together is shown in figure 1. After a 10-hour exposure the ozonated hexene caused as much as a 43 per cent stimulation of respiration of the lemons. A statistical analysis of the data showed that the ozonated hexene treatment was significantly different from the control at the 5 per cent level during the first 28 hours after the end of the treatment. The ozone alone seemed to cause an initial decrease in respiration followed by a small stimulation. The hexene appeared to slightly inhibit respiration. However, neither of these treatments was significantly different from the controls at the 5 per cent level any time during the experiment. All the treatments reached about the same respiration rate as the control fruit after 48 hours.

A citrus fruit during the growing season would not be subjected to a single exposure but in most cases successive exposures of air pollutants. Because of this it is pertinent to know the response of lemons to repeated exposures of both ozone alone and to ozonated hexene. The stage of development of the fruit might exert some influence on the respiratory response, so fruit of a mature size that were very dark green in color and fruit of a lighter color were subjected to the above gases.

Figure 2 A shows that lemon fruits exposed to successive treatments of ozone respond in much the same way to the second or third treatment as they do to the first. The only trend that suggests itself is that there may be a slightly greater response of the fruit to the ozone treatment after previous applications. The dark green lemons seemed to respond more to successive treatments than the riper fruit. A statistical analysis of the results showed that the respiration of the ozone treated lemons was significantly higher than the controls at the 1 per cent level. The respiration of the dark green fruit was not significantly different from the light green fruit the first day and only slightly different the second and third days at the 5 per cent level.

The response of these green lemons to ozonated hexene was much more striking than to ozone alone as shown in figure 2 B. The respiration rate of dark green lemons was stimulated even more after previously being
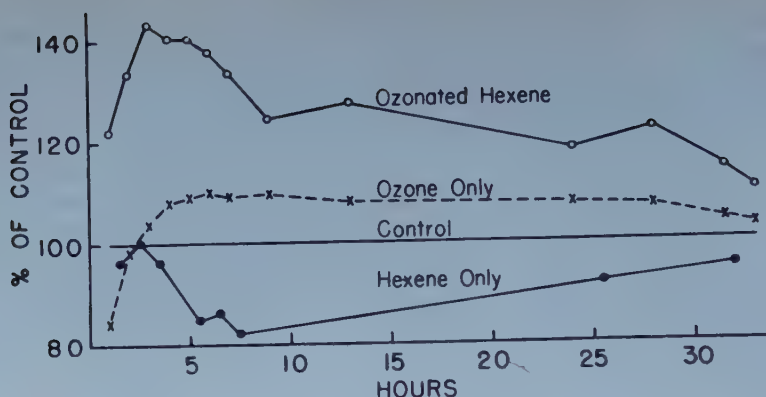
Figure 1. *The respiration rate of yellow, waxed lemons after a 10-hour treatment with various gases.* Gas concentrations: ozone 6.5 μmoles/mole of gas; hexene 5070 μmoles/mole of gas; final oxidant concentration with ozonated hexene (phenolphthalin reagent) 0.45 μmoles/mole of gas. Control fruit respiration (average) 19.4 mg. CO_2/kg. fr. wt./hr. Each point represents the average of 4 replications.

treated with the gas. This suggests that with the more immature fruit, the treatment brings about some change which causes it to respond more to subsequent treatments. Respiration of the ozonated hexene treatment was significantly higher than the controls at the 1 per cent level and the dark green fruit had a greater respiration than the light green fruit, which was significant at the 1 per cent level.

The above experiments establish the fact that lemon fruit respiration is stimulated by fairly long exposures (10—15 hrs.) to both ozone and ozonated hexene. The following experiments were devised to determine what effect short exposures to these gases might have on fruit respiration. The fruit used were of a light green color and the concentrations of gases were about the same as those used in the previous experiment (Figure 2). The treatments of ozone and ozonated hexene were for 1, 2 and 4 hours and the figures give the respiration rate after the end of the fumigations.

The ozone alone (Figure 3 A) caused a slight depression in the 1 and 2 hour treatments followed by a stimulation of respiration as in the previous experiments where longer fumigations were given. There appear to be no large differences between the shorter and longer treatments.

The ozonated hexene treatment (Figure 3 B) caused a marked stimulation after 1 hour of treatment and longer treatments appeared to cause an even greater stimulation. Thus again this combination of gases proved to be a more powerful stimulant than ozone given alone.
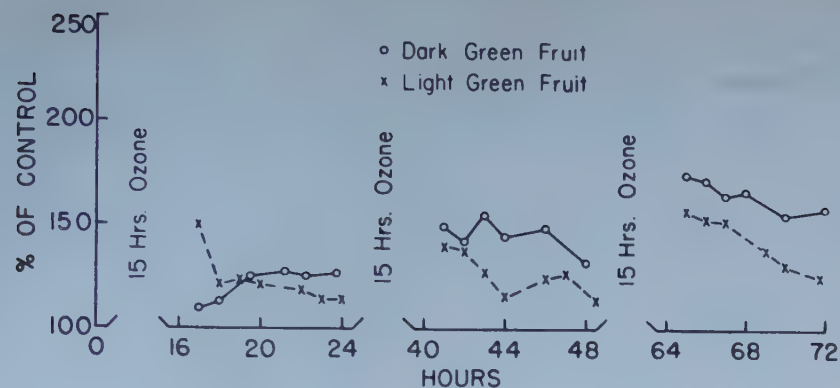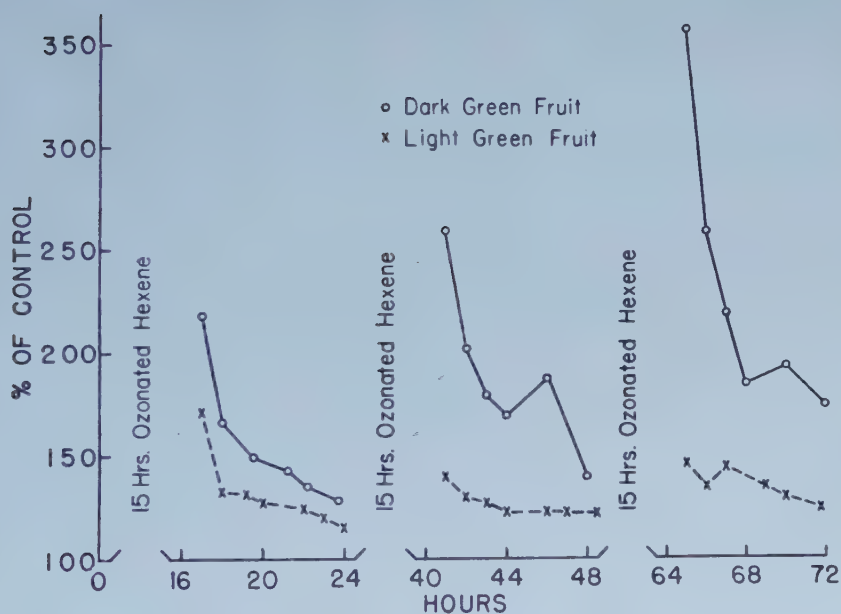
Figure 2. *The respiration rate of green lemons following 15-hour treatments of ozone or ozonated hexene given on successive days. Figure 2 A. The effect of ozone at a concentration of 1 μmole/mole of gas. Figure 2 B. The effect of ozonated hexene; concentrations of reactants* (calculated as though there were no reaction): ozone — 1 μmole/mole of gas; hexene — 7000 μmoles/mole of gas. Control fruit respiration (average for each day): Light green fruit — 17.1, 14.5, and 13.1 mg CO_2/kg. fr. wt./hr. for the first, second, and third days, respectively; Dark green fruit — 13.7, 12.1, and 11.6 mg. CO_2/kg. fr. wt./hr. for the first, second and third days, respectively. Each point represents the average of 4 replications.
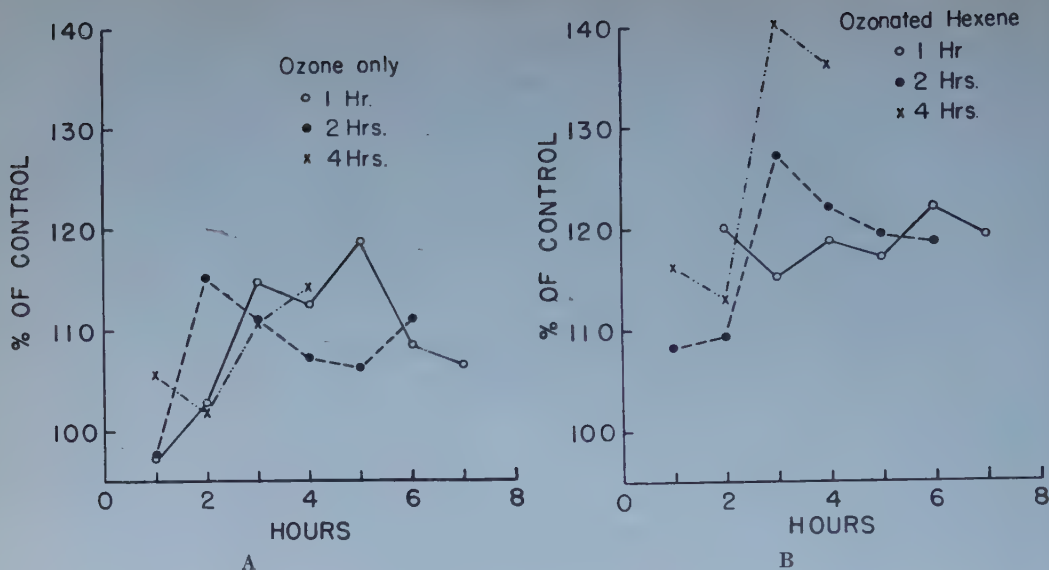
Figure 3. *The respiration of green lemons following treatments of ozone and ozonated hexene.* Figure 3 A. *The effect of ozone at a concentration of 1.2 μmoles/mole of gas.* Figure 3 B. *The effect of ozonated hexene; concentrations of reactants:* ozone — 1.2 μmoles/mole of gas; hexene — 7450 μmoles/mole of gas; final oxidant concentration (thiocyanate reagent) 0.19 μmoles/mole of gas. Control fruit respiration (average): ozone — 21.4 mg. CO_2/kg. fr. wt./hr.; ozonated hexene — 20.5 mg. CO_2/kg. fr. wt./hr. Each point represents the average of 4 determinations.

Discussion

These experiments have shown that both ozone and ozonated hexene have a stimulating effect on the respiration of lemon fruit. Ozonated hexene proved to be a more powerful stimulant than ozone by itself. The largest stimulation obtained with ozone alone was less than twice the respiration of the control (Figure 2 A), whereas the respiration of similar fruit treated with ozonated hexene gave stimulations up to $3\frac{1}{2}$ times that of the control (Figure 3 B). In all the experiments the actual oxidant concentration is much higher when ozone was used alone than after reaction with hexene. From the excess concentrations of hexene used there should have been practically no free ozone left uncombined. The measurements given in figure 3 support this contention since 1.2 μmoles of ozone/mole of gas were introduced into the reaction tube and the final oxidant concentration was only 0.19 μmoles/mole of gas which probably represents some intermediate oxidant product and not ozone (17).

The mechanism of action of these oxidants in producing a respiratory

stimulation remains to be investigated. If the effect is merely one of oxidation, then ozone alone should be superior to the ozonated hexene. However, the ozonated hexene may be able to enter the plant cells more readily because of a greater fat solubility, thereby making it a more effective oxidizing agent than ozone.

The respiratory response of the fruit appears to be somewhat dependent on the maturity of the fruit since the dark green fruit responded more to both ozone alone and when combined with hexene than the light green fruit (Figure 2). The yellow fruits used in Figure 1 were waxed so are not directly comparable with the green fruit. Bartholomew *et al.* (2) found that green (immature) Valencia oranges absorbed gaseous HCN more readily than mature fruit. Therefore, the greener lemons might absorb these gases more readily also.

The ozone content of the atmosphere in the Los Angeles area is reported to be abnormally high (1, 16), frequently being from 0.3 to 0.5 ppm and up to 1.0 ppm. The concentrations used in the ozone fumigations exceed this amount (2 to 3 times in the experiments reported in Figures 2 and 3, and nearly 10 times in the experiment reported in Figure 1). A direct comparison of the ozonated hexene concentrations used with those occurring in the Los Angeles atmosphere cannot be made since the exact hydrocarbons existing in the atmosphere are not known. However, the oxidant concentrations used were in the same range as often obtained there.

The question arises as to what effect these air pollutants might have on fruit growing on the tree in polluted atmospheres. If the citrus fruit is as susceptible at other stages of growth and the response is the same when the fruit is on the tree as when it has been picked, then there could be an excessive respiratory rate during periods of aggravated air pollution. The fruit would then consume an abnormal amount of carbohydrates, which if not used for growth, might cause retarded fruit development or a subnormal carbohydrate content.

Summary

The respiration of commercially mature picked lemon fruit can be markedly stimulated by treatment with a mixture of ozone and 1-hexene gases at low concentrations for as little as one hour. Longer treatments gave greater respiratory stimulations, as much as $3 \frac{1}{2}$ times the control. When ozone was used alone as the treatment, the lemon fruit respiration showed a smaller stimulation (on one occasion more than $1 \frac{1}{2}$ times the control). Hexene, when used alone, caused no change in the respiration rate. The

stage of ripeness had some effect on the magnitude of the fruit respiratory response particularly to ozonated hexene. Dark green fruit responded more than light green fruit, and yellow fruit responded the least.

References

1. Air Pollution Control District, County of Los Angeles. Second Tech. & Admin. Report on Air Pollution Control in Los Angeles County. 1950—51.
2. Bartholomew, E. T., Sinclair, W. B., & Lindgren, D. L.: Measurements on hydrocyanic acid absorbed by citrus tissues during fumigation. — Hilgardia 14: 373. 1942.
3. Claypool, L. L. & Keefer, R. M.: A colorimetric method for CO_2 determination in respiration studies. — Proc. Am. Soc. Hort. Sci. 27: 520. 1942.
4. Erickson, L. C. & Wedding, R. T.: Effects of ozonated hexene on photosynthesis and respiration of Lemna minor. — Am. Jour. Bot. 43: 32. 1956.
5. Green, J. & Johnson, A. H.: Effect of petroleum oils on the respiration of bean leaves. — Plant Physiol. 6: 149. 1931.
6. Haagen-Smit, A. J., Darley, E. F., Zaitlin, M., Hull, H., & Noble, W.: Investigation on injury to plants from air pollution in the Los Angeles area. — Plant Physiol. 27: 18. 1952.
7. — & Fox, M. M.: In L. C. McCabe. — Ind. Eng. Chem. 45 (Sept.): 111 A. 1953.
8. Homan, C.: Effects of ionized air and ozone on plants. — Plant Physiol. 12: 957. 1937.
9. Johnson, C. M. & Hoskins, W. M.: The relation of acids and peroxides in spray oils to the respiration of sprayed bean leaves and the development of injury. — Plant Physiol. 27: 507. 1952.
10. Knight, H., Chamberlain, J. C., & Samuels, C. D.: On some limiting factors in the use of saturated petroleum oils as insecticides. — Plant Physiol. 4: 299. 1929.
11. Koritz, H. G. & Went, F. W.: The physiological action of smog on plants. I. Initial growth and transpiration studies. — Plant Physiol. 28: 50. 1953.
12. Middleton, J. T., Kendrick, J. B., Jr., & Darley, E. F.: Air-borne oxidants as plant-damaging agents. — Proc. Third Natl. Air Poll. Symposium, Pasadena, California. April 18, 19, 20, 1955.
13. — Kendrick, J. B., Jr., & Schwalm, H. W.: Injury to herbaceous plants by smog or air pollution. — Plant Dis. Reporter 34: 245. 1950.
14. Minshall, W. H. & Helson, V. A.: The herbicidal action of oils. — Proc. Am. Soc. Hort. Sci. 53: 294. 1949.
15. Noble, W. M.: Pattern of damage produced on vegetation by smog. — Jour. Agr. & Food Chem. 3: 330. 1955.
16. Stanford Research Institute.: The Smog Problem in Los Angeles County. 1954.
17. Todd, G. W.: A modification of ferrous thiocyanate colorimetric method for determination of some atmospheric oxidants. — Anal. Chem. 27: 1490. 1955.
18. Wedding, R. T. & Erickson, L. C.: Changes in the permeability of plant cells to $P^{32}O_4$ and water as a result of exposure to ozonated hexene (smog). — Am. Jour. Bot. 42: 570. 1955.

The Base Curvature Response of Avena Seedlings to the Ultraviolet

By

GEORGE M. CURRY, KENNETH V. THIMANN,[1] and PETER M. RAY[2]

Harvard Biological Laboratories
Cambridge, Massachusetts
(Received April 16, 1956)

Introduction

The phototropic curvature of the Avena seedling comprises two components, those of the tip and of the base. The tip response is the more sensitive, requiring a minimum of only about 2 ergs/cm² for visible curvature, and less still with microscopic observation (Arisz 1915). This is the response which has been the more generally studied. The base response, which is actually given by the whole seedling, requires relatively high light quantities (van der Wolk 1911) of the order of hundreds ergs/cm², although these depend to some extent on the duration of the exposure (Arisz 1915). It apparently takes place more quickly than the tip response (Went 1925). During the course of an extended study of the spectral sensitivity of phototropism in Avena it was noted that the base response was given particularly well in the ultraviolet, and that in the shorter wavelengths of this region the plants were completely free from simultaneous tip curvatures. Mills and Schrank (1954) have also reported on curvatures of Avena caused by ultraviolet, and have noted that they appear first near the base. It was therefore decided to make a detailed study of the base response. Experiments on the spectral sensitivity of the tip response will be reported elsewhere.

[2] Society of Fellows, Harvard University.

Methods

Seeds of *Avena sativa* var. "Victory" were hulled, soaked in water for two hours, and set out on wet cellulose tissue in small vials, the seed just projecting over the lip of the vial. The rows of vials were placed in the dark room at 25° C and 85 per cent relative humidity, exposed again to red light for 2 hours and then left in darkness till 76 hours old. The red light for this purpose was of about 12 ft-candles and was provided by 2 fluorescent lamps mounted behind Corning signal red filters. The purpose of the red light exposure was to produce seedlings with meso-cotyls (or first internodes) approximately 1 cm. long.

For the UV exposure the seedlings (handled in a weak red or green light) were given measured doses of radiation from an H85C3 General Electric capillary mercury lamp, which operates under pressure. The spectral lines from this lamp were isolated by means of a Bausch and Lomb grating monochromator, the half band-width being about 3.3 mμ. Their intensities were measured with a thermopile and galvanometer, calibrated against a standard lamp from the National Bureau of Standards. After illumination the plants were shadowgraphed immediately and again 90 minutes later on the same film, using green light and Kodalith Ortho Type 2 film. The green light had no effect on the curvatures. The plants remained in the dark at 25° and were not moved between these two shadowgraph exposures. From the resulting images of the plants in the initial and final state the curvatures were measured with a goniometer.

General Nature of the Base Curvature

Figure 1 shows the curvatures resulting from unilateral exposure to approximately 600 ergs/cm² of radiation of wavelength 280 mμ. The position of the coleoptilar node is indicated by a tiny drop of lanoline applied prior to the exposure. It is clear that the final curvature is largely confined to the base of the plant, extending to the node and in most cases below it.
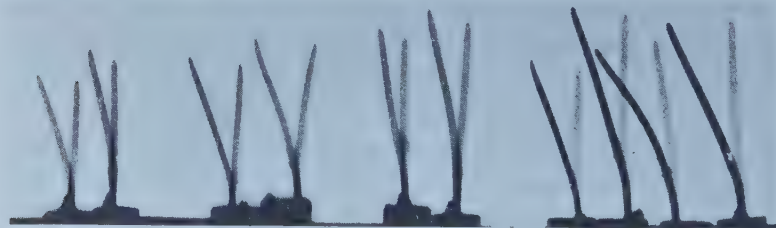

Figure 1. *Shadowgraphs showing initial and final positions* (after 90 minutes) *of plants receiving* (from the left side) *600 ergs/cm² at 280 mμ.* The age of the six plants on the left of the figure was 76 hours, and of the four on the right was 78 hours. The position of the coleoptilar node is shown by a tiny lanoline mark on the right side of each plant.

Shadowgraphs taken at 30 minute intervals show that the curvature appears within the first 30 minutes and begins along the entire length of the seedling. It increases to a maximum at about 90 minutes, though the upper part of the plant assumes a straighter appearance after the first 60 minutes. After the maximum the curvature usually regresses somewhat for at least the next 30 minutes.

Localization of the Sensitivity

For a study of the distribution of the sensitivity to UV, plants were lined up by using the 577 mμ mercury line together with a pair of glass filters transmitting yellow only, in order to eliminate second order UV coming through the monochromator at this wavelength. This light gave no measurable curvatures, either at tip or base, in control plants. Limited zones of the plants were then exposed to approximately 600 ergs/cm^2 of 280 mμ UV. This region was chosen because longer wavelengths elicit a marked tip curvature, superimposed on the base curvature, in plants whose tips are exposed to the radiation. These tip curvatures are similar to those caused by visible light, but from 290 to about 320 mμ they cannot be obtained without also getting some base curvature.

These experiments showed that if only 4 to 5 mm. of the tip is irradiated, the resulting curvature is about half that given by plants exposed all over, and in the early stages is more confined to the tip. Irradiation of 7 cm. of the middle of the coleoptile gives practically no response, while irradiation of the nodal region gives somewhat less than half the control response. Hence the tip region, as well as that around the node, can respond to UV by causing a *base* curvature.

It is suggestive to note that the region below the tip and that around the node are the two regions of the seedling which are elongating most rapidly at this age. This is shown clearly in Figure 2 (solid line) where the growth rate was determined by following small lanoline droplets over a period of 100 minutes, the plants being photographed at the beginning and end of the period with green light. Furthermore it was found that a 60 minute exposure of the plants to red light prior to the UV reduces the base curvatures subsequently obtained by about one half. This is in agreement with the observation that the growth rate in the nodal region and in the mesocotyl below, i.e. the region in which most of the base curvature takes place, is severely reduced by the red light. The changed growth pattern is shown by the dotted line in Figure 2, which was obtained on a plant in every way
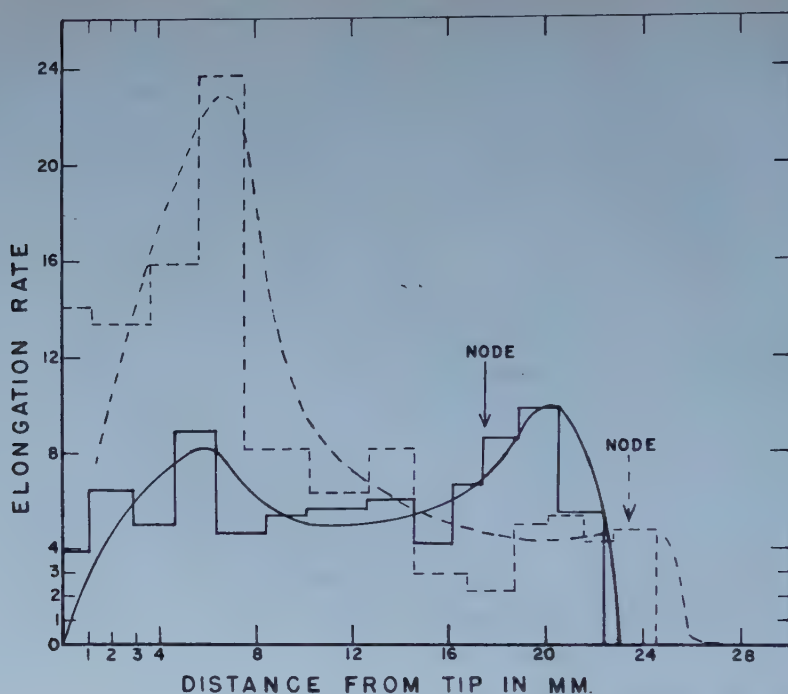
Figure 2. *Distribution of growth rate* (as % elongation in 100 minutes) *by zones along the Avena seedling.* Solid line, plant handled as in text. Dotted line, plant given an additional hour of red light just prior to measurement. The actual zone elongation measurements are shown in the histograms.

comparable to the other, but exposed to red light for one hour before the growth measurements.

The Base Curvature as a Function of the Radiant Energy Applied

Figure 3 shows the results of a single experiment involving 12 rows of 6 plants each. Each row was given a different dose of 280 mμ energy by maintaining the rows at a constant distance from the source and varying the time of exposure from 0 to 512 seconds. It should be mentioned here that in all cases in this work the I×t law seemed to be valid within the experimental error. It is clear that the curvature increases as a logarithmic function of the applied energy, finally reaching a maximum value at curvatures of 16 to 20 degrees. It should be noted that the control plants show variations (±3°) ofthe same order as those exposed to the UV, which in
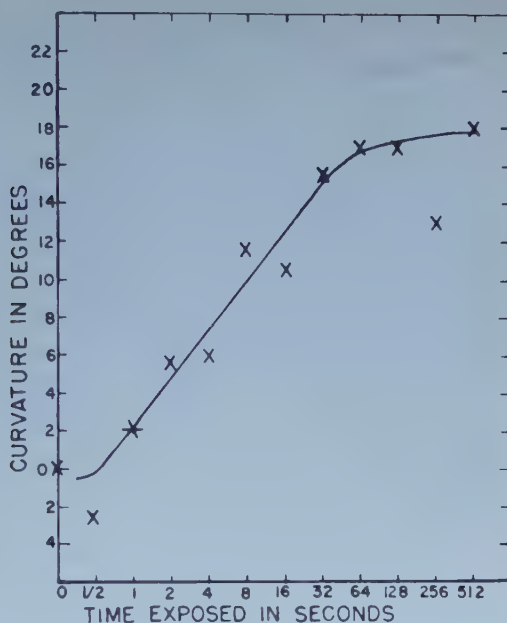
Figure 3. *Relation between exposure and base curvature.* Each second of exposure represents 55 ergs/cm² of 280 mμ. Each point is the mean of six curvatures.

each group show an everage deviation of ±2.4° from the group mean. This variation may therefore be attributed at least in part to nutations.

The Action Spectrum

Rows of four plants each were exposed to predetermined doses of UV light of various wavelengths. The plants were arranged behind a mask in such a way that 2 to 4 mm. of the tip of each received no UV light, so that the tip curvatures, which would have been caused by wavelengths above 290 mμ, did not complicate the results. The four plants in each row were staggered sufficiently so that there was no shadowing of one plant by another, all alignment being carried out in the light of the filtered yellow Hg lines.

The action spectrum is presented in Figure 4. All the points shown were obtained in a single experiment, numerous repetitions of which amply confirm the various features of the curve. From preliminary experiments the doses given here were selected so as to yield a curvature of approximately 8° at each wave length, a value low enough to avoid responses in the saturation region (cf. Figure 3). Of the mean curvatures obtained, 80 per cent fell in the range between 6.4° and 10.4°, the average of all the curvatures
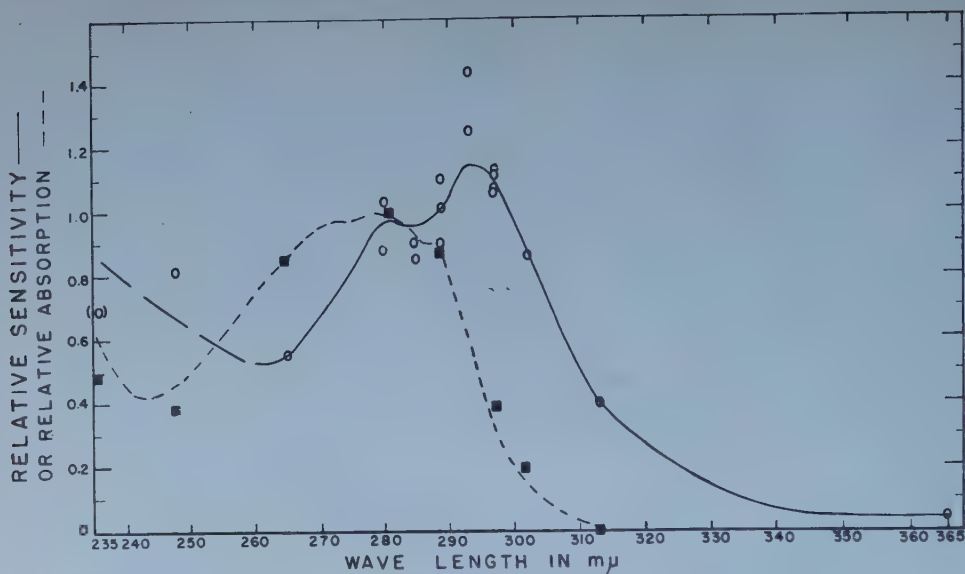
Figure 4. *Action spectrum for the base curvature* (circles and solid line). The ordinates give the reciprocal of the number of quanta needed for 8.6° curvature, relative to that at 289 mμ. The dotted curve is the absorption spectrum of IAA, the optical density at 280 mμ having been taken as 1.0. The black squares show the action spectrum for photo-oxidation of IAA solutions in vitro, the value at 280 mμ being taken as 1.0.

being 7.9°. From the mean of each row, the energy necessary to give a fixed response (8.6°) was calculated by a linear relationship. The data of Figure 4 indicate the reciprocal of the relative numbers of quanta necessary to give this response at each wave length, 289 mμ being taken as unity. The absolute energy required at 289 mμ for this response is approximately 320 ergs/cm².

In Figure 4 the points at 236 and 248 mμ are least accurately determined because the output of the lamp is so low in this region that the energy determinations become very difficult. Similarly, the points at 285 and 293 mμ cannot be weighted heavily, since these wavelengths are between emission lines of the lamp, where the energy determinations suffer in accuracy. Qualitatively, however, they are doubtless correct in showing that the sensitivity at 293 mμ is well above, and that at 285 mμ well below, the reference level at 289 mμ.

The action spectrum shows a rather broad peak with a maximum in the region just below 297 mμ and a shoulder on the lower wavelength side at 280 mμ. It falls off quite steeply on both sides, rising again below 260 mμ. On the higher wavelength side the relative sensitivity is very low above 330 mμ. A base response can still be obtained at 365 mμ, but energy of

another order of magnitude is required. However, plants exposed to 365 mμ without shading the tips exhibit well defined tip curvatures at very much lower energies (ca. 10 ergs/cm²).

The Base Response with Indole-Acetic Acid

It was found that exposure to UV immediately after decapitation of 2—3 mm. of the tip gave less than half the response of the intact plants. Attempts were therefore made to remove the auxin from seedlings by successive decapitations and to replace it by allowing indole-acetic acid (IAA) to diffuse into the cut tips. It was found that if the IAA was given sufficient time to diffuse into the seedling the sensitivity could be greatly increased. Figure 5 shows a typical experimental set of plants. Approximately 3 mm. of the tip of each plant was removed initially and the primary leaf pulled out. The plants were left for 90 minutes and then a second decapitation (1—2 mm.) performed. Agar blocks containing IAA (500 μg/l in 1.5 per cent agar) were applied to the four on the left. After 90 minutes the blocks were removed, a third decapitation made, and the seedlings then given approximately 2300 ergs/cm² of 297 mμ UV. They were shadowgraphed at once and again 90 minutes later. Numerical data of a similar experiment are given in Table 1. It is evident that IAA largely restores the sensitivity to UV.

The above procedure, which was arrived at after numerous preliminary experiments, is the most desirable because the controls so treated show a complete loss of sensitivity, and the effect of the IAA is clear. It should be mentioned that the third decapitation is found to be necessary, because small curvatures result if this step is omitted. Such curvatures probably result

Table 1. *Response of coleoptiles to paired exposures of monochromatic UV, 297 mμ, either applied to intact plants* (with tips shielded) *or immediately after third decapitation.* (100 minutes between decapitations; IAA 500 μg/l. in 1.5 % agar; 6 plants per group). Blocks in some cases removed just before exposure with no change in results.

Group	Decapitation	Treatment after 2nd Decapitation	Exposure in ergs/cm²	Curvature in degrees
1	(None, intact)		278	8.8
2	» »		348	10.4
3	3 ⎫	⎧ IAA blocks on for	640	4
4	3 ⎭	⎩ 75 minutes above node	1,920	9
5	3 ⎫	⎧ Cut to 15 mm., then	640	4
6	3 ⎭	⎩ IAA blocks on for 30 min.	1,920	7
7	3 ⎫	⎧ No blocks on,	640	0.5
8	3 ⎭	⎩ (controls for 3 and 4)	1,920	0
9	3 ⎫	⎧ Cut to 15 mm., no blocks	640	0
10	3 ⎭	⎩ on (controls for 5 and 6)	1,920	0

29

Figure 5. *Shadowgraphs of curvatures toward ultraviolet light* (297 mμ) *of decapitated plants illuminated after auxin application* (*cf.* Figure 1). The four plants on the right received blocks containing IAA for 90 minutes before exposure; the four plants on the left, plain agar. (Blocks removed before photographing).

from regeneration occurring in the 90 minute interval after the second decapitation.

The restoration of sensitivity by IAA takes time. If the IAA is allowed to diffuse into the tissues for only 30 minutes, the UV curvatures are largely confined to the upper five mm. of the coleoptile; efter 60 minutes' diffusion they extend approximately 10 mm. down the plant, and after 90 minutes they usually reach the nodal region. It seems therefore that only the IAA which has penetrated well into the plant at the time of exposure to UV is effective in the increase of sensitivity, and that the rate at which the sensitivity moves downward, about 10 mm. per hour, is precisely that at which IAA is known to move in the coleoptile (van der Weij, 1932). Careful examination of Figure 5 shows that the controls have ceased to grow after the third decapitation, whereas the plants with added IAA are elongating at a rate approximately half that of intact plants. Again it is clear that the base response sensitivity is correlated with growth rate at the time of exposure. The decapitations involve the removal of one of the most actively growing regions (*cf.* Figure 2), perhaps accounting in part for the overall decrease in sensitivity.

An important fact observed in numerous experiments is that addition of IAA *after* UV exposure does not restore sensitivity. Thus in plants decapitated only once, IAA blocks applied immediately after the exposure do not appreciably affect the resulting small curvatures. If the plants are successively decapitated as described above, but IAA blocks applied *after* the UV exposure (2000 ergs/cm²), no curvatures develop. This behavior contrasts with that reported for phototropism in visible light (*cf.* Went and Thimann, 1937, Chap. 10). It is also not what would be expected from Mills and Schrank's finding (1954) that a previous exposure to UV reduced the subsequent curvature of coleoptiles when auxin was applied unilaterally. However, the high

doses of broad-spectrum UV employed by these workers no doubt had an effect different from that reported on here.

An extensive series of experiments such as that shown in Figure 5 were performed at various wave lengths. It was found that the action spectrum for this process corresponded quite well with that found in intact plants. It is difficult to determine this precisely since the variations involved in applying blocks, etc. are compounded with the variations already inherent in the intact plants. One feature, however, is quite definite. The response obtained at 297 mµ is of the same order as that at 280 mµ. The significance of this will be shown below. There is also a definite small response, though requiring much more energy, at 365 mµ.

Action Spectrum for the in vitro Destruction of Indoleacetic Acid

The fact that applied IAA can make decapitated plants sensitive to UV suggests that the observed curvatures are due to photo-inactivation of IAA by ultraviolet light. The sensitivity of natural auxins to UV has been known since the work of Laibach and Maschmann (1933), and of Burkholder and Johnston (1937), and several authors have suggested that this photo-inactivation could be responsible for the effects of UV on plants (*e.g.* Popp and McIlvaine, 1937; von Denffer and Schlitt, 1951; Mills and Schrank, 1954). To compare the process of photo-oxidation with the phototropic response, the action spectrum for the photo-oxidation of pure IAA in aqueous solution was determined. It was found that to obtain appreciable photo-oxidation, the energy doses required were several thousand times greater than those effective in phototropism. The procedure used was as follows: A quartz cuvette of 1 cm. light path, containing 1.7 ml of aqueous IAA solution (12 mg./l), was placed close up to the monochromator so that the entire light output (slit width 1 mm.) traversed the solution. The geometry was such that a minimum volume of the solution remained unilluminated. After a period of irradiation chosen to give approximately 20 per cent destruction of IAA (30 min. at 280 mµ, 47 min. at 265 mµ) 1 ml of the irradiated sample was added to 2 ml of Salkowski reagent (Gordon and Weber, 1951) made up and kept in the dark (*cf.* Platt and Thimann, 1956). The optical density of this solution at 530 µ was determined with a Beckman DU spectrophotometer after 30 minutes in the dark; a similar assay was performed on the unirradiated IAA solution. Separate experiments showed that the disappearance of IAA during irradiation, determined in this way, follows approximately first-order kinetics. The irradiation times, at different wavelengths, at which exactly 20 per cent of the Salkowski color would have disappeared (t_{20} %)

could thus be estimated by a log-linear interpolation (or extrapolation) from the measured values. Multiplying each t_{20} % by the relative quantum intensity at that wavelength (using the same energy calibration as for experiments on phototropism) gives the relative quantum dose required to cause 20 per cent destruction of IAA $(Q_{20}$ %).[3] The reciprocals of these values are a measure of the relative photodestructive effectiveness of different wavelengths of ultraviolet.

In two experiments the decrease of auxin activity in the same solutions was followed by bio-assay on Avena coleoptile sections in the standard manner. The auxin activity fell off approximately proportional to the decrease in Salkowski color throughout.

The results with the Salkowski reagent are plotted (as black squares) in Figure 4. It will be seen that with the possible exception of those at 236 and 248 μ, which are uncertain for the same reasons as with the action spectrum of phototropism, the points fall almost exactly on the absorption spectrum.

Discussion

The resemblance between the action spectrum for phototropic base curvature and the absorption spectrum of indoleacetic acid, shown as a dotted line in Figure 4, is striking. It extends not only to the asymmetric double peak but to the steepness of the slope on either side and the minimum at around 250 mμ. Two interpretations of this resemblance are possible. One is that it is coincidental; many substances have similar spectra, those of diphenylbutadiene and some colorless carotenoid derivatives being particularly suggestive. The spectrum does not agree, however, with those of phytofluene or phytoene; the peak of the latter (in hexane) lies at 286 mμ, but the wide shoulder on the long wavelength side at about 297 mμ (Claes 1954) differentiates it sharply from Figure 4. It should be noticed also that the complete absence of a peak at 260 mμ excludes the participation of riboflavin. Riboflavin shows another strong peak at 365 mμ where again the sensitivity is very low.

The other and more attractive interpretation is that the action spectrum is in fact that of IAA itself. However, a difficulty with this interpretation is that the action spectrum is clearly shifted about 12 mμ toward the visible. The shift is the more significant since the *in vitro* photo-oxidation does not

[3] This Q_{20} % is equal to the absolute quantum dose multiplied by a geometrical constant, due to the fact that for practical reasons the cuvette was placed 0.5 cm from the monochromator exit lens, while the energy calibrations were made where the plants were placed, namely some 16 cm from the exit lens.

show it. Reference to Figure 4 shows that for photo-oxidation 297 mμ is about one third as effective as 280 mμ, while for base phototropism 297 mμ is even a little more effective than 280 mμ. The same relationship was observed in decapitated plants supplied with IAA. The magnitude of the shift in spectrum is probably too great to be ascribed to a simple solvent effect, and moreover, there is a change in the form of the curve around the peak. A logical hypothesis is, then, that the IAA is in some form of *chemical combination.*

If such a compound exists, three deductions may be made about it. Firstly, since its absorption of UV affects growth, it must be a compound in which the IAA remains biologically active. Secondly, the far higher UV dosages needed to inactivate IAA in simple solution show that when in the compound the IAA must be sensitized to UV light. Thirdly, since it was shown above that the action spectrum for decapitated plants treated with IAA is roughly the same as for intact plants, it follows that IAA applied externally must be readily converted to the same compound as is naturally present in the plant. Further study will be needed to decide whether a compound of IAA having these properties actually exists in the plant.

It may be added that the base curvatures produced by the longer wavelengths at 365 mμ and above strongly resemble the "second positive" curvatures caused by high intensity visible light. The two phenomena are, indeed, very likely continuous.

Summary

1. Avena seedlings exposed unilaterally to low dosages of ultraviolet show positive phototropic curvature which develops over the whole length of the plant and soon becomes mainly located in the base. Both the tip and the region around the node can detect the UV, and the sensitivity of these zones is approximately proportional to their relative growth rates.

2. The curvature is proportional to the logarithm of the applied energy and at wavelength 280 mμ it reaches a saturation value of 16—20° at about 3000 ergs/cm^2.

3. Plants which have been repeatedly decapitated become completely insensitive to the UV, but the sensitivity is largely restored by applying indoleacetic acid in agar blocks *before* exposure to UV.

4. The action spectrum of the curvature has been determined. It shows peaks at 280 and 297 mμ. The wavelengths absorbed by riboflavin are relatively ineffective.

5. The action spectrum for the photoinactivation of pure indoleacetic acid

has been determined under comparable conditions. It resembles exactly the absorption spectrum of this substance in solution.

6. It is tentatively suggested that the UV curvatures may be due to a light-sensitive, growth-controlling chemical compound of IAA, in which the absorption spectrum is slightly modified and shifted about 12 mμ towards longer wavelengths.

References

Arisz, W. H.: Untersuchungen über den Phototropismus. — Rec. trav. bot. Neérl. 12: 44. 1915.

Burkholder, P. R. & Johnston, E. S.: Inactivation of plant growth substance by light. — Smithsonian Misc. Coll. 95, no. 20, 1937.

Claes, H.: Analyse der biochemischen Synthesekette für Carotinoide mit Hilfe von Chlorella-Mutanten. — Zeit. für Naturforschung 9 b: 461. 1954.

von Denffer, D. & Schlitt, L.: Blühförderung durch Ultraviolettbestrahlung. — Naturwiss. 24: 564. 1951.

Gordon, S. A. & Weber, R. P.: Colorimetric estimation of indoleacetic acid. — Plant Physiol. 26: 192. 1951.

Laibach, F. & Maschmann, E.: Ueber den Wuchsstoff der Orchideenpollinien. — Jahrb. wiss. Bot. 78: 399. 1933.

Mills, K. S. & Schrank, A. R.: Electric and curvature responses of the Avena coleoptile to unilateral ultraviolet irradiation. — J. Cell. Comp. Physiol., 43: 39. 1954.

Platt, R. S. Jr. & Thimann, K. V.: Interference in Salkowski assay of indoleacetic acid. — Science 123: 105. 1956.

Popp, H. W. & McIlvaine, H. R. C.: Growth substances in relation to the mechanism of the action of radiation on plants. — J. Agr. Res. 55: 931. 1937.

van der Weij, H. G.: Der Mechanismus des Wuchsstofftransportes. — Rec. trav. bot. Neérl. 29: 379. 1932.

Went, F. W.: Concerning the difference in sensibility of the tip and base of Avena to light. — Proc. Kon. Akad. Wetensch. Amsterdam, 29: 185. 1925.

— & Thimann, K. V.: Phytohormones. — New York, MacMillan, 1937.

van der Wolk, P. C.: Investigation of the transmission of light stimuli in the seedlings of Avena. — Proc. Kon. Akad. Wetensch. Amsterdam, 14: 327. 1911.

The "Rhizosphere Effect" of Graminaceous Plants in Virgin Soils

III. Comparison with the Effect of Other Plants

By

Helge Gyllenberg and Pauli Hanioja

Department of Microbiology, University of Helsinki, Finland
(Received April 25, 1956)

In an earlier report from this laboratory (Gyllenberg, Hanioja, and Vartio-vaara, 1954) it was shown that the vegetation influences the quantity and quality of the bacterial flora in virgin soils. Additional investigations (Gyllenberg, 1955, 1956) have provided evidence to show that graminaceous plants promote the development of certain types of soil bacteria. To obtain a more clear-cut picture of these phenomena, additional experiments have been performed to compare the effect of graminaceous plants with that of other plants. Hereby particular attention was paid to the typical vegetations of certain common forest types in Finland.

Methods

Quantitative and qualitative data were recorded according to the methods described by Gyllenberg (1955, 1956). As in the earlier reports the "rhizosphere effect" is expressed as the "r/s-ratio", where (r) is the count obtained from the rhizosphere sample, and (s) the count from the corresponding control sample. Accordingly, a r/s-ratio lower than 1 indicates that the count from the rhizosphere sample is lower than that from the control. In such cases the plant roots obviously have exerted an inhibiting effect on the bacteria.

The figures presented below concern only bacteria. Although the actinomycetes were considered during the course of the investigation, the data concerning them have been omitted because they seem not to be especially noteworthy.

The investigation comprised the following forest types: Oxalis-Myrtillus Type (OMT), birch-stand; Myrtillus Type (MT), spruce-stand; and Vaccinium Type (VT), pine-stand. The following plants were selected as test plants: seedlings of birch, spruce and pine, red whortleberry (Vaccinium vitis-idaea), Deschampsia, and Pleurozium.

Rhizosphere as well as control samples were collected from two different depths of soil: the A_0-horizon and the B-horizon. The investigation was carried out during the years 1954 and 1955, and the samples were taken in July—August.

Results

The Quantitative "Rhizosphere Effect". The main quantitative results are presented in Table 1. These figures show that the roots of trees and shrubs exert a negative "rhizosphere effect" in the A_0-horizon. In the deeper B-horizon, on the other hand, a distinct promoting effect was found with the seedlings of birch and spruce, and with red whortleberry in the MT soil. The fact that the effect of red whortleberry remained much weaker in the B-horizon of the VT soil than in the MT soil may indicate that also pine behaves as birch and spruce, i.e. that the difference found between the effects of pine and spruce in the B-horizon of VT and MT soil, respectively, was rather due to soil conditions than to the plants themselves.

These results show that soil bacteria (or certain types of them) are inhibited by the woody parts of the roots of trees and shrubs (it can be mentioned here that actinomycetes seem to be affected to a lesser degree by the inhibiting principle), but that the root hairs exert a promoting effect.

The roots of Deschampsia showed in both MT and OMT soil, and in both the horizons investigated, a significant promoting effect on the bacteria. With this plant also the r/s-ratios were higher in the B-horizon than in the A_0-horizon, but the difference was much smaller than found with the trees and shrubs. Moreover, the positive effect of Deschampsia was in all samples

Table 1. *The r/s-ratios for different plants in OMT, MT, and VT soil.*

Soil	Plant					
	Birch	Spruce	Pine	Vaccinium	Deschampsia	Pleurozium
OMT, A_0	0.1	—	—	—	15.9	3.4
B	11.1	—	—	—	28.4	—
MT, A_0	—	0.2	—	0.5	46.6	11.4
B	—	22.4	—	10.0	70.0	—
VT, A_0	—	—	0.04	0.3	—	1.7
B	—	—	0.9	1.6	—	—

Table 2. *The r/s ratios for different plants, showing the effect on the different nutritional groups of bacteria.*

Nutritional groups	Trees and shrubs			Deschampsia	
	VT	MT	OMT	MT	OMT
BA	1.3	17	8	106	21
Aa	0	0	0	36	18
AV	0.63	38	17	104	25

distinctly higher than the effects of other plants. With Pleurozium a rather slight promoting effect was found in the A_0-horizon of all soils.

The Qualitative Effect of Different Plants. The nutritional characteristics of isolates from each A_0-sample (some 25—30 cultures) were tested according to the scheme of Gyllenberg (1956). On the basis of this testing the isolates were grouped as three "nutritional groups": (Ba) bacteria showing definite growth in the basal medium, (Aa) bacteria requiring amino acids as essential nutrients, and (AV) bacteria requiring both amino acids and B-vitamins as essential nutrients.

The nutritional testing, the results of which are given in Table 2, revealed the interesting fact that the Aa group of bacteria was definitely inhibited in all soils by the woody parts of the roots of trees and shrubs, but distinctly promoted by Deschampsia. The both other groups (Ba and AV) were promoted by trees and shrubs in the MT and OMT soils, but more strongly so by Deschampsia. In the VT soil the trees and shrubs exerted a very slight if any effect on these nutritional groups.

These results show that the bacteria of the Aa group are greatly dependent on the conditions prevailing in the rhizosphere of graminaceous plants. This dependence is especially significant since these bacteria are inhibited by certain other plants dominating the forest vegetations.

Discussion

A recent investigation of Metz (1955) shows that the roots of several grass plants exert a strong inhibiting effect on the rhizosphere bacteria of other plants and on "non-rhizosphere" bacteria. The topical organisms were not characterized, but it can be supposed that the inhibiting effect was confined to a certain group of bacteria. Our investigation included no detailed experiments on the inhibition of the Aa group bacteria by the woody parts of the roots of trees and shrubs, but our results agree with the considerations of Metz indicating that the composition of the "rhizosphere flora" can be influenced also by an inhibiting effect on some types of soil bacteria.

As to the two other groups of soil bacteria (Ba and AV) it seems that there is no certain difference between the "rhizosphere effect" of the plants investigated. In this connection it must be noted that the effect on the Ba and AV groups seemed to be of the same order of magnitude in all samples. This may suggest a certain relationship between both these groups. As shown by Gyllenberg (1956) the AV group organisms can take advantage of the B-vitamins synthetized by the vitamin-independent types (belonging to groups Ba and Aa). It is well understandable, therefore, that an increase in the numbers of the latter types results in a corresponding increase in the number of the vitamin-dependent types also. Accordingly, the results obtained in the present investigation provide additional evidence to show that the "rhizosphere effect" is primarily confined to bacteria of the Ba and Aa types, which effect, in turn, may give rise to a secondary one, the enumeration of vitamin-requiring bacteria (group AV).

Lochhead and Thexton (1947) have found that in cropped soils the "rhizosphere effect" is confined to bacteria which are capable of maximal growth with inorganic nitrogen and without an addition of B-vitamins, and to those which are dependent on amino acids. These types obviously are related to the bacteria belonging to the Ba and Aa groups according to our grouping. Moreover Lochhead and Thexton reported that bacteria requiring preformed vitamins are relatively less abundant in rhizosphere soil than in "non-rhizosphere" soil. Our experimental material does not permit definite conclusions as to the relative abundance of vitamin-requiring bacteria in "non-rhizosphere" and rhizosphere soil, respectively, but it seems evident that in forest soils also, amino acid secretion by plant roots gives rise to the primary positive "rhizosphere effect".

The figures in Table 2 also show that soil conditions influence the composition of the rhizosphere flora and the "rhizosphere effect". The highest r/s-ratios were obtained in the MT soil, the values in the OMT soil were lower, and in the VT soil only a very slight if any effect on the bacterial groups in question was found.

Summary

The "rhizosphere effect" of trees (seedlings), shrubs, graminea, and mosses have been investigated in three different forest soils. The results show that the woody parts of the roots of trees and shrubs exert an inhibiting effect on the bacteria which are dependent on amino acids in their nutrition. The growth of these bacteria, which can be considered as the types most distinctly dependent on rhizosphere conditions, is however, promoted also by trees

and shrubs in deeper soil horizons. The "rhizosphere effect" of graminea was, however, higher than that of trees and shrubs in all samples. With mosses a slight promoting effect was found in the A_0-horizon.

References

Gyllenberg, H.: The "rhizosphere effect" of graminaceous plants in virgin soils. — Physiol. Plant. 8: 644. 1955.

—   The "rhizosphere effect" of graminaceous plants in virgin soils. II. Nutritional characteristics of non-sporogenous bacteria associated with the roots. — Physiol. Plant. 9: 119. 1956.

—   & Hanioja, P. & Vartiovaara, U.: Observations on the composition of the microbial population in some virgin soils (English Summary). — Acta Forest. Fen., 62: 1. 1954.

Lochhead, A. G. & Thexton, R. H.: Qualitative studies on soil microorganisms. VII. The "rhizosphere effect" in relation to the amino acid nutrition of bacteria. — Can. Jour. Res. C, 25: 20. 1947.

Metz. H.: Untersuchungen über die Rhizosphäre. — Arch. f. Mikrobiologie, 23: 297. 1955.

Physico-Chemical Studies of the Phosphorylating Enzymes of *Oscillatoria princeps*

By

Jerome F. Fredrick

Research Laboratories, New York Division, Dodge Chemical Company,
New York 23, N.Y.
(Received April 28, 1956)

Introduction

The two enzymes, *phosphorylase* and *branching enzyme*, involved in the synthesis of polyglucosides from glucose-1-phosphate in the blue-green alga, *Oscillatoria princeps*, were first described by Fredrick (1951). In a series of papers, some of the physical constants (Fredrick 1954), as well as the chemistry of these enzymes (Fredrick and Mulligan 1955, Fredrick 1955), were elaborated.

More recently, interest has centered on the enzymes of a low-temperature mutant, *LTV*, of this alga (Fredrick 1952, 1953). The differences reported in the type of polyglucoside synthesized by the *LTV* strain (Fredrick 1952, 1953 b) were thought to have arisen from a basic difference in the enzymes. To elucidate these differences, the new techniques of paper electrophoresis were applied to these studies. A qualitative study of these enzymes by electrophoretic methods, showed a gross similarity of the patterns, and hence, no apparent differences between the phosphorylating enzymes of normal, *N*, and *LTV* strains (Fredrick and Mancini 1955). An extension of these studies on a quantitative basis was therefore, undertaken in order to ascertain whether differences of a physico-chemical nature existed on a molecular level between the enzymes of *N* and *LTV* strains.

Experimental

Samples of *purified mixtures* of phoshorylase and branching enzyme from *N* and *LTV* strains of *Oscillatoria princeps* were prepared as described by Fredrick and Mancini (1955). The enzymatic activity of these samples was tested as described (Fredrick and Mancini 1955), and they were then subjected to dialysis for 12 hours at 5° C. against a series of buffers. The buffers were: veronal-acetate, pH 8.6, ionic strength 0.11; phosphate, pH 6.8 ionic strength 0.1; acetate, pH 5.4, ionic strength 0.1; acetate, pH 4.7, ionic strength 0.1.

After dialysis, samples of the enzymes were withdrawn and their activities tested to insure that no loss of activity had occurred at the various pH levels. The enzymes were then studied in their respective buffers by means of the electrophoresis apparatus used previously by Fredrick and Mancini (1955), which was based on that of Kunkel and Tiselius (1951). All runs were made at 23° Centigrade and at a potential of 4 volts per centimeter and 4 milliamperes.

The electropherograms were dyed with bromphenol blue as described (Fredrick and Mancini 1955), and then scanned by means of an Analytrol Integrator (Spinco Division, Beckman Instrument Company, California, U.S.A.). The curves obtained were compared, and the mobilities, "u", calculated by the method of Lederer (1955). *Zones III* and *IV* (cf. Fredrick and Mancini 1955) were retested for *phosphorylase* and *branching enzyme* activity after electrophoresis.

In order to compensate for the phenomenon of *electroosmosis,* a correction factor based on the mobility exhibited by a spot of dextran was used to correct the apparent "u" values of the enzymes et each pH (Kunkel and Tiselius 1951, Jermyn and Thomas 1953).

The *isoelectric point* for each enzyme was obtained by interpolation of the curve of the plot of "u" against pH (Cohn and Edsall 1943). This method has been used extensively for free electrophoresis and works equally well for paper electrophoresis (Schwarz 1951, McDonald et al 1951). After correction for electroosmosis, the isoelectric points so obtained, compare favorably with those determined by free electrophoresis (Kunkel and Tiselius 1951, Lederer 1955).

Results

The scanned patterns at pH 8.6, 6.8, 5.4 and 4.7 for the purified enzyme mixtures from *N* Oscillatoria princeps are shown in Figure 1. The peaks corresponding to *Zones I, III* (phosphorylase), *IV* (branching enzyme), and *VI* (a salt effect) of the electrophorograms described in a previous paper by Fredrick and Mancini (1955), are indicated in the pen-drawn curves from the scanned electropherograms. The results from *LTV* strains were identical with the patterns shown.

The actual electropherograms at pH 6.8, 5.4, 5.1 and 4.7 are shown in Figure 2 after staining with bromphenol blue. It can be seen that as the pH approaches that of the isoelectric point of branching enzyme (Figure 2, pH 5.1), very little migration is manifested by *Zone IV.*
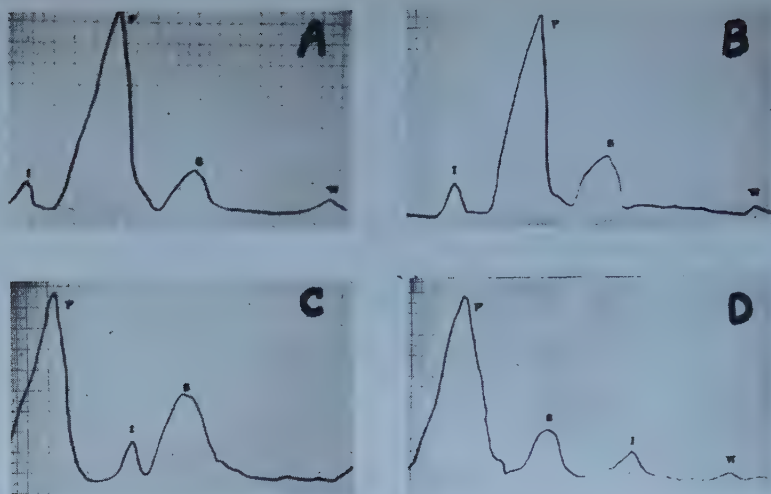
Figure 1. *Pen-drawn patterns from scanned electropherograms of enzyme mixtures from N. Oscillatoria princeps.* A. in veronal-acetate buffer, pH 8.6; B. in phosphate buffer. pH 6.8; C. in acetate buffer, pH 5.4; D. in acetate buffer, pH 4.7 All buffers at ionic strength 0.1. P, phosphorylase; B, branching enzyme; I, point of application of enzymes to paper strip; VI, salt effect (see text).
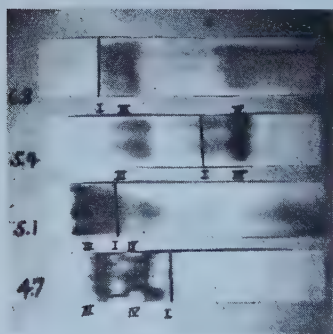

Figure 2. *Electropherograms of enzyme mixtures from N Oscillatoria princeps at various pHs.* Zone III, phosphorylase; Zone IV, branching enzyme; I, point of application to the filter paper strip. Note that at pH 5.1 there is practically no migration of Zone IV even after 12 hours, from the point of application. This is near the isoelectric point of branching enzyme (see text).

Figure 3. *Interpolation to obtain the iso electric point of the phosphorylating enzymes of N Oscillatoria princeps.* Ordinate, mobility as $u \times 10^{-5}$ cm.²/volt second; Abscissa, pH as measured with a glass electrode at 23° Centigrade. The isoelectric points are indicated by pI at the point of "zero" mobility. P, phosphorylase; B, branching enzyme. These points have been corrected for electroosmosis (see text).
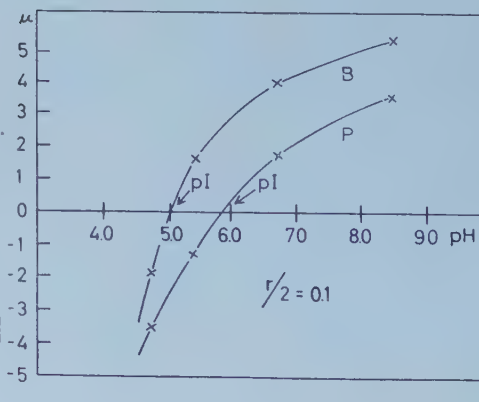
Table 1. *Isoelectric points* [1] *of phosphorylases and branching enzymes of N and LTV strains of O. princeps.*

Enzymes	Strain	
	N	LTV
Phosphorylase............	5.85—5.90	5.92—6.00
Branching enzyme......	5.00—5.15	4.95—5.10

[1] Corrected for electroosmosis.

Figure 3 shows the interpolation of the curve of "u"/pH to obtain the isoelectric points for the phosphorylase and the branching enzyme of N strains. The isoelectric points for *LTV* strains are shown in the combined figure, Table I. All these points have been corrected for electroosmosis as described.

Briefly, it should be pointed out (see Figure 1) that in all instances, there appeared to be a *greater* absolute concentration of phosphorylase to branching enzyme in N and *LTV* strains. Work is now in progress to determine the concentrations of the phosphorylases and branching enzymes in these strains.

Another observation is confirmed by the isoelectric points of phosphorylase and branching enzyme. As can be seen in Table 1, the pIs for branching enzyme and phosphorylase are quite close. This would serve to explain the inability to separate these two enzymes completely. As stated in previous papers, there appears to be a coprecipitation of both enzymes by the chemical methods used to isolate them (Fredrick 1951, 1953, 1955).

Discussion

A summary of the data to this point, is shown in Table 2. It can be seen that no essential points of difference exist between the phosphorylases and branching enzymes of N and *LTV* strains of *Oscillatoria princeps*. The enzymes appear to be identical in both strains.

It has been indicated that the polyglucoside synthesized by LTV strains of this alga, is practically devoid of branching (Fredrick 1952, 1953 b). It was thought, at first, that this difference is polyglucoside reflected a *deletion* or *alteration* of the branching enzyme in *LTV* strains. However, subsequent work showed that branching enzyme *existed* in *LTV* strains (Fredrick 1953 b, 1954), and that this enzyme was *identical* with that present in N strains (Fredrick 1954, Fredrick and Mulligan 1955). The possible presence of an

Table 2. *Summary of physico-chemical properties of the phosphorylating enzymes of N and LTV Oscillatoria.*

Properties	Phosphorylase		Branching Enzyme	
	N	LTV	N	LTV
pH optima[1]	7.0	7.0	6.8	6.8
K$_s$[2]	0.08 M	0.08 M	1.58 M	1.60 M
pI[3]	5.88	5.96	5.07	5.02
Action[4]	same as LTV	same as LTV	—	—

[1] Fredrick (1956).
[2] Michaelis-Menten Constant (Fredrick 1954).
[3] Fredrick (1956), Corrected for electroosmis.
[4] Fredrick and Mulligan (1955).

inhibitor specific for branching enzyme in *LTV* strains was also investigated and disproved (Fredrick 1951, 1952, 1953 b). The present study has failed to reveal any difference on a molecular level between the branching enzymes of *N* and *LTV* strains.

In view of the identity of *N* and *LTV* branching enzymes, an explanation is still lacking for the synthesis of a less-branched sugar by *LTV* strains of this alga. It is hoped that differences in the *absolute concentration* of this enzyme in these two strains will become apparent in subsequent studies now in progress in this laboratory. As was indicated (Fredrick and Mulligan 1955, Fredrick and Mancini 1955), such differences in concentration would be compatible, when viewed in conjunction with the mode of action of branching enzyme (Fredrick and Mulligan 1955, Fredrick 1955), with the genetic phenomenon of mutation.

Conclusions

Quantitative electrophoresis of the enzymes involved in the synthesis of polyglucosides in *N* and *LTV* strains of *Oscillatoria princeps* indicated that the enzymes were identical in both strains.

The isoelectric points obtained by interpolation from the curve of electrophoretic mobility/pH, showed no essential differences between the phosphorylases of *N* and *LTV* strains, or between the branching enzymes of these strains.

It is suggested that the mutant's enzymes are molecularly identical with the normal's, and that differences in the polyglucoside synthesized by these two strains must be due to a difference in *concentration* of branching enzyme.

References

Cohn, E. J. & Edsall, J. T.: Proteins, Amino Acids and Peptides. — Rheinhold Publishers, N.Y., 1943.

Fredrick, J. F.: Preliminary studies on the synthesis of polysaccharides in the algae. — Physiol. Plant. 4: 621. 1951.

— A polysaccharide variant of Oscillatoria princeps. — Physiol. Plant. 5: 37. 1952.

— Induction of polysaccharide variants in Oscillatoria by low temperatures. — Physiol. Plant. 6: 96. 1953.

— Branching characteristics of Oscillatoria polyglucosides. — Physiol. Plant. 6: 100. 1953.

— Kinetics of polysaccharide formation in extracts of Oscillatoria princeps. — Physiol. Plant. 7: 182. 1954.

— Proposed structure for straight and branched polymers of glucose. — Physiol. Plant. 8: 288. 1955.

— & Mulligan, F. J.: Mechanism of action of branching enzyme from Oscillatoria and the structure of branched dextrins. — Physiol. Plant. 8: 74. 1955.

— & Mancini, A. F.: Paper electrophoresis patterns of enzymes involved in polyglucoside synthesis in O. princeps and its low temperature strains. — Physiol. Plant. 8: 936. 1955.

— Enzyme content of plant tissues and organs. — In Handbook of biological data (to be published by National Academy of Sciences-National Research Council, 1956).

Jermyn, M. A. & Thomas, R.: Reduction of liquid flow in paper electrophoresis. — Nature 172: 128. 1953.

Kunkel, H. G. & Tiselius, A.: Electrophoresis of proteins on filter paper. — J. Gen. Physiol. 35: 89. 1951.

Lederer, M.: Introduction to paper electrophoresis and related methods. — Elsevier Press, New York. 1955.

McDonald, H. J., Urbin, M. C. & Williamson, M. B.: Ionography: some aspects of ion migration on filter paper in an electric field. — J. Colloid Sc. 6: 236. 1951.

Schwarz, V.: Microelectrophoresis on filter paper. — Nature 167: 404. 1951.

Breaking the Dormancy of Prosopis Seeds

By

A. K. KHUDAIRI

Department of Botany College of Arts and Sciences, Baghdad, Iraq
(Received May 2, 1956)

Introduction

Many members of the family Leguminosae have seeds which possess hard testas (Crocker and Barton, 1953). *Prosopis stephaniana* (Willd.) Spreng. [*P. farcta* (Banks et Soland.)] Eig. is a small plant belonging to the sub-family Mimosoideae. It grows in Iraq as a woody xerophytic shrub in desert, open field, pasture and open pine forest habitats up to an altitude of 1000 metres. It dries out above the ground in autumn but from the old axes young shoots appear again in spring. Very few young seedlings are to be seen in the field.

As far as the author is aware no previous work has been done on the physiology of *Prosopis stephaniana,* though preliminary investigations showed that its seeds are dormant and possess a hard testa. A study was accordingly made of the absorption of water by Prosopis seeds, of various methods of breaking dormancy in them, and of the effect of temperature on germination of such treated seeds.

Material and Methods

Prosopis stephaniana fruits were collected from the vicinity of Baghdad. Healthy seeds were selected from these fruits and used in lots of 50 seeds per treatment. Concentrated sulphuric acid, acetone, ethylene chloride, chloroform, 90 per cent ethyl alcohol, 20 per cent sodium hydroxide solution,

and carbon tetrachloride were tried as chemical means of breaking the dormancy of the seeds; filing, freezing at $0°$ C., boiling water, soaking in water at $60°$ C., drying in an oven at $60°$ C., and autoclaving were tried as mechanical means of breaking the dormancy. The duration of each treatment is indicated in the appropriate part of the text.

The treated seeds were placed on moistened filtered paper in Petri dishes, 50 seeds per dish, and incubated in a germinating oven at a particular temperature. The emergence of the radicle from the seed coat was considered to indicate the beginning of germination.

Experimental Results

I. *Anatomical Structure of the Seed Coat*

Microtomed sections of Prosopis testa showed that it is composed of an outer layer of elongated palisade cells with thick walls and neck-like protrusions. This palisade layes is covered by a thick cuticle (Figure 1). The middle region of the testa is composed of approximately 25 layers of sclerenchyma cells. The inner region is composed of a single layer of thin-walled cells.

II. *Effect of Chemical Treatments on Dormancy*

In this experiment, 50-seed lots were soaked for 30 minutes in each of the following chemicals; concentrated sulphuric acid, 20 per cent sodium hydroxide solution, 90 per cent ethyl alcohol, carbon tetrachloride, chloroform, and ethylene chloride. The seeds were then washed thoroughly with water and allowed to germinate on moistened filter paper in Petri dishes. The dishes were incubated in a germinating oven at $25°$ C., germination counts being made after 5 days. The results of this experiment are tabulated in table 1. Concentrated sulphuric acid proved to be the most effective of all the chemicals tried. Soaking seeds in acetone for 30 minutes proved a successful treatment for softening the hard seed coat. It is worth mentioning, however, that soaking in acetone for periods longer than 30 minutes gave higher values of germination, *viz.*, 20 per cent. Treatment with 20 per cent sodium hydroxide solution, 90 per cent ethyl alcohol, chloroform, carbon tetrachloride, or ethylene chloride proved non-effective in inducing germination.

As a further proof of this marked effect of concentrated sulphuric acid, Prosopis seeds were soaked in the acid for 20 minutes, washed, and allowed to germinate on moistened filter paper at $26°$ C. Germination counts were made over a period of 12 days. Controls were soaked in water for comparison.
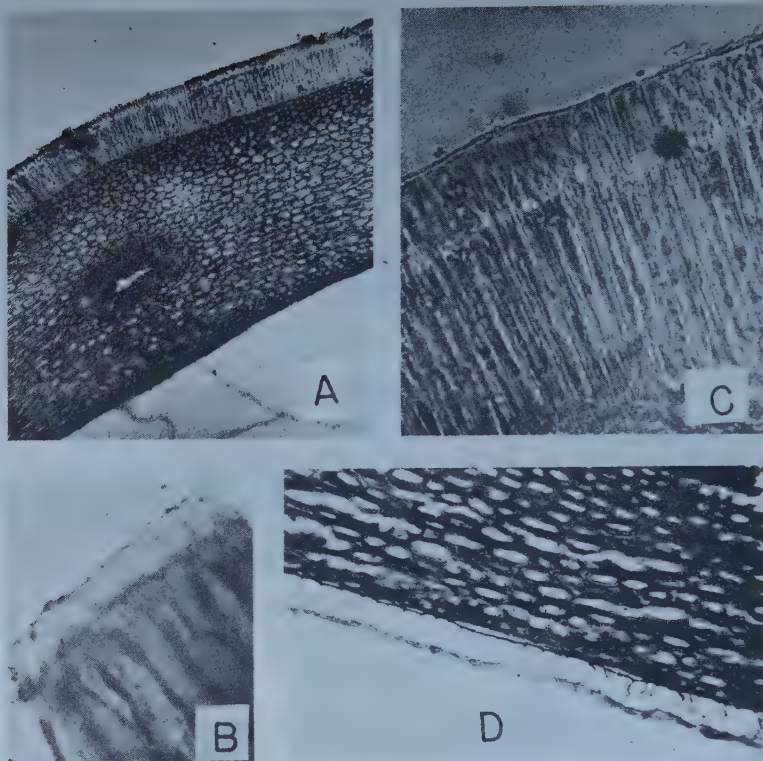
Figure 1. *Transverse section through Prosopis testa. A* shows the whole testa. *B* the thick cuticle. *C* the palisade sclerenchymatous layer, with no intercellular spaces. *D* several layers of the middle sclerenchyma region and the lower thin-walled inner layer.

Table 1. *Effect of chemical treatment on germination of Prosopis seeds.* The seeds, 50 per treatment, were soaked for 30 minutes in different chemicals washed with water, and then germinated on moistened filter paper in Petri dishes in a germinating oven at 25° C. The percentage of germination was observed 5 days after treatment.

Treatment	Initial weight of seeds (gms.)	Weight of seeds after 24 hrs. (gms.)	Water absorbed (gms.)	% water absorbed[1] in 24 hrs.	% germination (after 5 days)
Conc. H_2SO_4	3.93	6.06	2.13	54.5	72
NaOH (20 %)	3.42	3.80	0.38	11.1	6
Acetone	3.63	4.44	0.81	22.5	14
Carbon tetrachloride	3.52	3.55	0.03	0.77	2
Chloroform	3.80	3.93	0.13	3.15	2
Ethylene chloride ...	3.98	4.15	0.17	4.27	2
Ethyl alcohol (90 %)	3.82	4.25	0.43	11.25	4
Water (control)	3.86	4.08	0.22	5.7	6

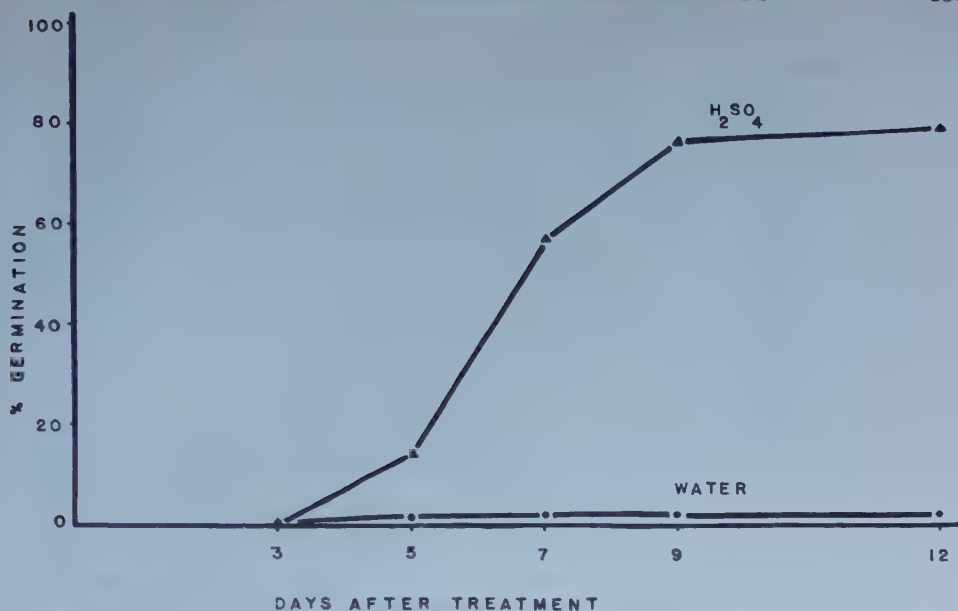[1] % water absorbed = water absorbed per 100 gm. initial weight of seeds.

Figure 2. *Germination of Prosopis seeds within 12 days of treatment.* 100 seeds were treated with concentrated sulphuric acid for 20 min., washed with water, and then germinated in Petri dishes at 24° C. Another 100 seeds were left intact but soaked in water as a control.

The results, as presented in Figure 2, show that treatment with concentrated sulphuric acid induced a steady increase in percentage germination with lapse of time. It was also thought advisable to study the effect of various soaking intervals on germination and the length of Prosopis radicles. This was carried out by soaking the seeds in concentrated sulphuric acid for 5, 15, 30, and 60 minutes, and then allowing the treated seeds to germinate at room temperature (36° C.). Germination counts and measurements of radicle length were carried out 4 days after the treatment. The results of this experiment are presented graphically in Figure 3. The result which stands out clearly in Figure 3 is that soaking the seeds in concentrated sulphuric acid for a period of 30 minutes seems to be optimal for breaking of dormancy and consequently of germination as well as for the length of radicles produced, though a slight increase in radicle length was observed in seeds that had been soaked for 60 minutes.

III. *Effect of Mechanical Treatment on Dormancy*

In this experiment, lots of 50 seeds each were also used. These seeds were either filed, frozen, or heated according to the scheme described earlier. The results are represented in table 2, from which it may be seen that:
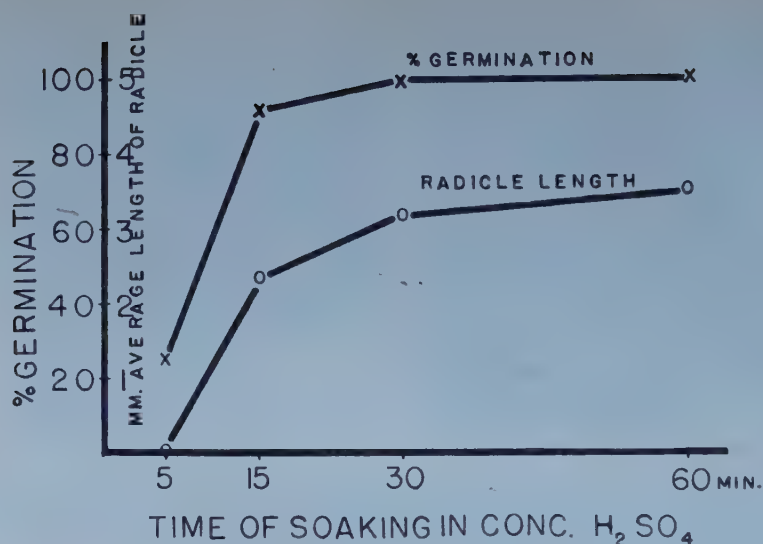
Figure 3. *Treatment with concentrated sulphuric acid for different intervals of time.* Fifty seeds were used in each treatment. Seeds were germinated at 36° C. Readings were made after 4 days.

1. When the seeds were filed they started to germinate within the first 48 hours of incubation, 82 per cent germination being obtained 5 days after treatment. Intact seeds, on the other hand, gave no more than 6 per cent germination by the end of the experimental period.

Table 2. *Effect of mechanical treatments for breaking the dormancy of Prosopis seeds.* Fifty seeds in each treatment were germinated on moistened filter paper in a germinating oven at 25° C.

Treatment	Duration of treatment	Initial weight of seeds (gms.)	Weight of seeds after 24 hrs. (gms.)	Water absorbed after 24 hrs.	% water absorbed	% germination after (5 days)
Intact	—	3.86	4.08	0.22	5.7	6
Hot water (60° C.) ..	15 min.	3.94	4.27	0.33	10.7	4
Hot water (60° C.) ..	30 min.	3.80	4.02	0.22	4.74	2
Boiling water	5 min.	3.63	5.20	1.57	43.7	36
Boiling water	15 min.	3.97	6.60	2.63	66.3	34[1]
Dry oven (60° C.) ...	15 min.	4.10	4.15	0.05	1.22	2
Dry oven (60° C.) ...	30 min.	4.05	4.07	0.02	0.49	2
Freezing, 0° C.	40 min.	3.67	3.72	0.15	1.36	4
Autoclave 20 lb./sq. in	20 min.	3.67	9.37	5.70	155.5	6[2]
Filed	—	3.50	8.12	4.62	129.5	82

[1] Seedlings had much smaller radicles than those boiled for 5 minutes.
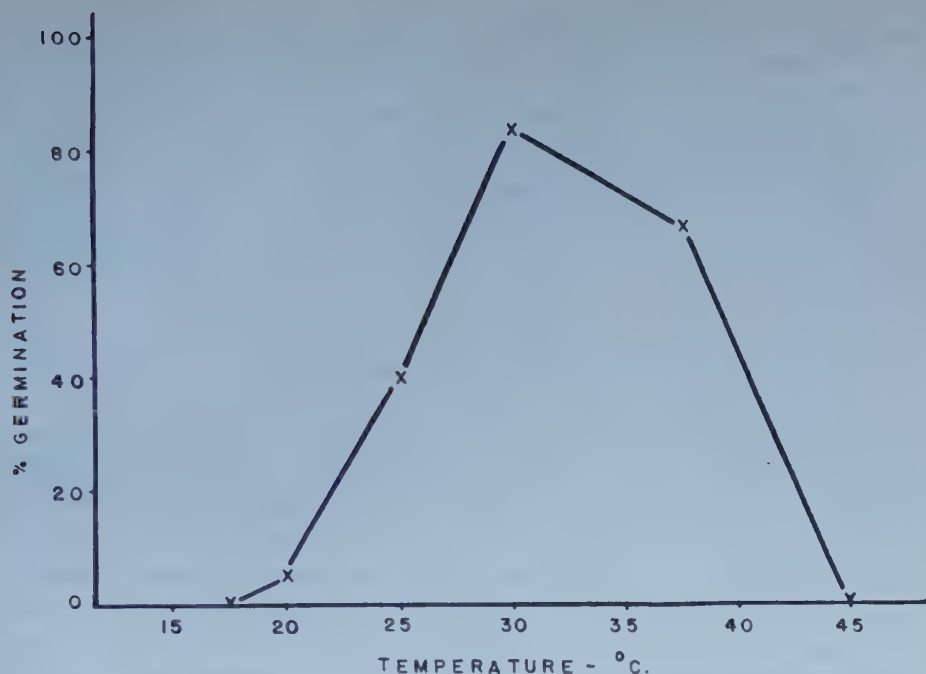[2] Seeds were mechanically damaged during treatment.

Figure 4. *Relationship between temperature and percentage germination.* Fifty filed seeds per treatment were used. Germination counts were made 5 days after treatment.

2. Boiling the seeds in water for 5 minutes gave 36 per cent germination. Fifteen minutes of boiling gave a similar percentage of germination but the radicles were much shorter than those of seeds boiled for 5 minutes.

3. Autoclaving for 20 minutes at 20 lb. per sq. inch damaged the seeds mechanically.

4. Heating in an oven at 60° C., freezing at 0° C., or soaking in warm water (60° C.) did not break the dormancy of the seeds.

IV. *Effect of Temperature on Germination of Chemically or Mechanically Treated Seeds*

Seeds were either filed or soaked for 30 minutes in concentrated sulphuric acid and then allowed to germinate, after washing in the latter case, at the following temperatures: 20, 22.5, 25, 27, 30, 37, and 45° C., for 5 days. The results are presented in Fig. 4 for the filed seeds. The optimum temperature for germination of Prosopis seeds was found to be between 27 and 30° C.

V. *Absorption of Water by Treated Seeds*

In all previous experiments it was observed that chemically or mechanically treated seeds were swollen owing to the absorption of water. This may be accounted for by assuming that these treatments caused the removal, of the thick layer of cuticle. Impermeability to water by the hard seed coat is thus the evident cause of dormancy in these seeds. It might be questioned, however, whether water is the only limiting factor in the germination of Prosopis seeds.

The non-treated (intact) seeds absorbed 5.7—9.5 per cent of water in 24 hours, calculated on the basis of the initial weight of the seeds (Tables 2 and 3). Soaking the seeds in water for 10 days, increased the amount of water absorbed to 30 per cent. The greatest amount of water absorbed in 24 hours was 155.5 per cent when the seeds were autoclaved for 20 minutes at 20 lb. per sq. inch, but the seeds were mechanically damaged by this treatment. Filed seeds absorbed 129.5 per cent of water in a period of 24 hours. Boiling the seeds for 15 minutes led to absorption of water to 66.3 per cent, while 43.5 per cent of water was absorbed when seeds were boiled for 5 minutes (Table 2). Seeds treated with concentrated sulphuric acid for 30 minutes absorbed 54.5 per cent water in 24 hours. Acetone-treated seeds absorbed 22.5 per cent of water in 24 hours (Table 1).

The smallest amounts of water absorbed were observed when the seeds were either dried in an oven at 60° C., frozen at 0° C., or treated with carbon tetrachloride (Tables 1 and 2).

The results presented in tables 1, 2 and 3 indicate that there seems to be some relation between the amount of water absorbed and the percentage of germination, except in the case of filed seeds. Table 3 shows that seeds treated with concentrated sulphuric acid gave 92 per cent germination at the end of 30 days while only 26 per cent of the filed seeds had germinated in the same

Table 3. *Water absorption and germination of Prosopis seeds.* Fifty seeds were used per treatment. Germinated at room temperature (18° C.) in Petri dishes on moistened filter paper.

Treatment	Duration of treatment	Water absorbed by seeds (gms)	% water absorbed	% germination after 30 days
Soaked in water	24 hrs.	0.374[1]	9.5	6
Soaked in water	10 days.	1.197	30.0	6
Con. H_2SO_4	10 min.	1.910[1]	37.5	24
Con. H_2SO_4	20 min.	2.513[1]	68.3	92
Filed from side	—.	4.133[1]	110.0	26

[1] Measurement after 24 hours.

Table 4. *Relation between water absorption and germination of filed Prosopis seeds and seeds treated with concentrated sulphuric acid.* Triplicates of 50 seeds were used for each treatment. The seeds were germinated in Petri dishes at 28° C. Percentages of germination and water absorption were measured 48 hours after treatment.

Seed conditions	Seeds treated with concentrated sulphuric acid for 20 mins.	Filed seeds
Dry weight in gms.	4.338	4.289
Weight after treatment in gms.	4.193	—
% loss of weight	3.57	—
Weight 48 hrs. after treatment ..	9.930	10.700
Water uptake in 48 hrs. in gms.	5.774	6.411
% water absorbed in 48 hrs. ...	133.6 ± 3.5	149.5 ± 1.8
% germination	71.6 ± 9.1	35.3 ± 10.8

time interval. It is to be observed that the amount of water absorbed by the filed seeds was much greater than that absorbed by the acid-treated seeds.

In order to investigate this peculiar behaviour of the filed seeds, a comparison was made of the percentage of germination after 48 hours of filed seeds with those soaked for 30 minutes in concentrated sulphuric acid. The results are given in Table 4. These results confirm the above findings, viz., that seeds treated with sulphuric acid gave much higher percentage germination than filed seeds although the latter absorbed much more water than the former.

Discussion

Barton (1947) has studied the germination of seeds of some leguminous plants. With *Prosopis velutina* she was able to get 100 per cent germination by filing the seeds or shaking them for 20 minutes in a glass bottle, while intact seeds gave only 30 per cent germination. She explains her results by suggesting that such mechanical treatment increased the permeability of the seed coat to water. She also suggested that soaking the seeds in absolute alcohol for 72 hours brings about almost the same result.

Watson (1948), using some members of the sub-family Papilionoideae, found no relation between the structure of the testa and the germinative power of the seeds.

The results reported in the present paper show a general correlation between the amount of water absorbed by *Prosopis stephaniana* and their rate of germination. Thus germination was high whenever the amount of water absorbed was relatively high, except in the case of seeds treated with concentrated sulphuric acid which gave much better germination than filed

seeds although the latter absorbed more water than the former during the same period of time.

This discrepancy could be explained on the basis of the fact that treatment in concentrated sulphuric acid softens the whole seed coat, thus leading to an increased oxygen exchange, or that the softening of the seed coat by treatment with sulphuric acid facilitates the emergence of the radicle more than does their filing. The fact that seeds treated with concentrated sulphuric acid lose about 3.6 per cent of their weight through this treatment (Table 4) may be taken as circumstantial evidence supporting this view.

The beneficial results obtained through seed treatment with concentrated sulphuric acid, acetone, and boiling water may be due to the removal of the thick cuticle of the seed coat. Similar observations were reported by Anderson *et al.* (1953) who found that immersion of okra seeds in acetone for 30 minutes favours germination. They suggested that acetone may reduce the quantities of fatty materials in the seed coat.

From the foregoing discussion of the results it may be concluded that the dormancy of *Prosopis stephaniana* seeds is due to the thick cuticle. The removal of this inhibitor (thick cuticle) increases the permeability of the testa to water and helps in the emergence of the radicle.

Temperature plays an important role in the germination of *Prosopis* seeds; the optimum temperature for germination lies between 27 and 30° C. Higher or lower temperatures than the optimum slow down germination regardless of the treatment.

Summary

1. *Prosopis stephaniana* seeds collected from the vicinity of Baghdad were found to be dormant. The intact seeds gave no more than 6 per cent germination at the optimum temperature. They have a hard seed coat.

2. Several chemicals were used in an attempt to break the dormancy of the seeds. Concentrated sulphuric acid proved to be the most effective of those tried.

3. The optimum temperature for germination lies between 27 and 30° C. Temperatures higher than 40° C. or lower than 20° C. markedly retard germination.

4. In the first 24 hours after treatment, filed seeds absorbed 110 per cent water (calculated on the basis of air-dry weight of the seeds), boiled seeds absorbed 66 per cent water, and seeds soaked in concentrated sulphuric acid absorbed 54—68 per cent water. Seeds frozen at 0° C. and seeds treated in an oven at 60° C. did not absorb any substantial amount of water.

5. The percentage of germination seems to be closely correlated with the

amount of water absorbed except in the case of concentrated sulphuric acid treated seeds. Consideration of the pertinent results suggests that germination can be dependent not only on the permeability of the seed coat to water but also on the softening effect of the seed coat which may lead to easier emergence of the radicle.

6. The removal of the thickened cuticle by some chemical or mechanical means used in this investigation resulted in a breaking of dormancy of *Prosopis* seeds.

References

1. Anderson, W. H., Carolus, R. L. & Watson, D. P.: Germination of Okra seeds as influenced by treatment with acetone and alcohol. — Proc. Amer. Soc. Hort. Sci, 62: 427. 1953.
2. Barton, Lela V.: Special studies on seed coat impermeability. — Contrib. Boyce Thompson Inst, 14: 355. 1947.
3. Crocker, W. & Barton. L.: Physiology of seeds. — Chronica Botanica Co., Walthom, Massachusetts. 1953.
4. Watson, D. P.: Structure of the testa and its relation to germination in the Papilionaceae Tribes Trifoliae and Loteae. — Ann. Bot. n. s., 12: 385. 1948.

Nutritional Requirements of Some Marine Fungi

By

Ulla Gustafsson and Nils Fries

Institute of Physiological Botany, Uppsala
(Received July 5, 1956)

Recent investigations have shown that the number of fungi growing in sea water is much greater than was earlier assumed (*e.g.* Barghoorn and Linder 1944, Harder and Uebelmesser 1955, Höhnk 1955, Vishniac 1955). Thus, numerous species of Phycomycetes, Ascomycetes and Fungi imperfecti have been described which are obviously confined to marine habitats. Except for some data published by Barghoorn and Linder (1944), very little is known about the physiology of these fungi. The present investigation aims at throwing some light upon the nutritional requirements of some marine Ascomycetes and Fungi imperfecti, which grow and sporulate on decaying wood.

Material and Methods

The following ten species were collected and grown in pure culture:

Ascomycetes: *Halophiobolus opacus* Linder, *H.* cfr. *salinus* Linder, *Pleospora purpurascens* R. Sant., *Trematosphaeria orae-maris* (Linder) R. Sant., *T. thalassica* R. Sant., *Sphaerulina longirostris* R. Sant., *S. orae-maris* Linder, and *Ceriosporopsis maritima* (Linder) R. Sant.

Fungi imperfecti: *Helicoma maritimum* Linder and *Diplodia orae-maris* Linder.

We profited from the continuing experiments of Dr. R. Santesson, who since 1953 has had a number of boards of different varieties of wood, partly submerged, in the sea at Kristineberg Marine Biological Station at the Swedish West Coast in order to study wood-decaying halophilic fungi. Most of the species mentioned originated from these test boards; a few species were taken from drift-wood. All determinations were made by Dr. Santesson, who will soon publish elsewhere a detailed account of the taxonomy and ecology of these species.

All of the Ascomycetes were isolated from ascospores and the Fungi imperfecti from pycnidial conidia. The spores were transferred as aseptically as possible to malt-agar plates made up with sea water (2.5 per cent malt extract and 1.5 per cent agar-agar). Contrary to the statements of Barghoorn and Linder (1944) contaminating bacteria did not cause much trouble and, when present, could be successfully fought down with chloramine.

The experiments were performed in 100 ml. Erlenmeyer flasks (Pyrex) at +25° C, a temperature which according to Barghoorn and Linder (1944) is optimal for marine fungi of this type. Usually each flask contained 25 ml. of the following nutrient solution: glucose 10 g., asparagine 1.0 g., Na_2SO_4 0.28 g., $Na_2HPO_4 \cdot 2 H_2O$ 2.25 g., KH_2PO_4 1.0 g., $CaCl_2$ 0.1 g., $MgCl_2 \cdot 6 H_2O$ 0.41 g., $MnSO_4 \cdot 4 H_2O$ 4.43 mg., $ZnSO_2 \cdot 7 H_3O$ 4.05 mg., ferricitrate 5.31 mg., citric acid 5.31 mg., and distilled water 1000 ml. pH was after autoclaving 7.0. This solution is identical with "Medium A2+B" described by L. Fries (1955), except that ammonium tartrate and thiamin were omitted. Because of the rather strong buffering capacity of the solution, pH changes during mycelial growth rarely exceeded 1 unit. The growth was estimated by determining the dry weight of mycelium produced during 14 days. The mean values of the tables represent the average of series each of which consisted of eight cultures.

Experiments

Surprisingly enough, most species proved to be almost indifferent to the salts contained in the sea water. Like *Pleospora purpurascens* of Table 1 they produced the same or almost the same growth in distilled water as in sea water with malt extract added as the only supplementary nutrient. Only *Halophiobolus opacus* failed to grow without sea water, a finding, which agrees with the observations of Barghoorn and Linder (1944), whereas *Ceriosporopsis maritima* responded in an almost opposite way. Both species grow almost permanently submerged in their natural habitats.

Some further tests indicated that *Halophiobolus opacus* produced good growth if sea water was exchanged for a solution of $CaCl_2$, NaCl, $MgCl_2$, and Na_2SO_4 in concentrations corresponding to those of the sea (ZoBell 1946, p. 21).

Table 1. *Growth of three species of marine fungi in a 0.5 % malt extract solution made up with distilled water or with sea water.* The total salt concentration of the sea water was c. 3 %.

Species tested	Dry weight of mycelium in mg.	
	Distilled water	Sea water
Halophiobolus opacus	0.2 ± 0	6.2 ± 0.6
Pleospora purpurascens	5.5 ± 0.7	4.5 ± 0.3
Ceriosporopsis maritima	20.7 ± 0.5	7.0 ± 0.9

Table 2. *Growth of three species of marine fungi in the synthetic solution with different nitrogen sources.* Each flask was also supplemented with 1 mg of yeast extract (Difco).

Species tested	Dry weight of mycelium in mg.		
	Asparagine 1 g./l.	$(NH_4)_2HPO_4$ 1.1 g./l.	KNO_3 1.7 g./l.
Pleospora purpurascens	6.5 ± 0.4	7.7 ± 0.2	5.8 ± 0.2
Helicoma maritimum	99.3 ± 3.5	27.3 ± 1.6	16.4 ± 0.6
Diplodia orae-maris	54.0 ± 4.2	15.9 ± 1.8	30.5 ± 2.2

As to the availability of different sources of nitrogen it is worth noting, that all ten species grew more or less vigorously with any of the tested nitrogen compounds, viz. asparagine, diammonium phosphate, and potassium nitrate. Asparagine proved to be the best nitrogen source in most cases, as exemplified by *Helicoma maritimum* in Table 2. *Diplodia orae-maris* diverges from all the other species tested by growing considerably better with nitrate than with ammonium.

With asparagine as a source of nitrogen the effect of thiamin (vitamin B_1) was tested and compared with that of yeast extract. Five species, *viz. Halophiobolus opacus, Pleospora purpurascens, Trematosphaeria orae-maris, T. thalassica,* and *Sphaerulina orae-maris,* required thiamin, at least for good growth, the others are obviously thiamin-autotrophic. Yeast extract may further increase or reduce the growth rate (Table 3).

Finally, the power of utilizing different sugars as carbon sources was investigated. As a source of nitrogen, asparagine, which contains carbon, was exchanged for potassium nitrate. In Table 4 three species exemplify the response to the six sugars tested. It seems particularly interesting that galactose and lactose, which are poor carbon sources for most fungi (cp. Lilly and Barnett 1951), can be utilized by most of the marine species of this investigation. Only the two *Trematosphaeria* species and probably also *Halophiobolus opacus* failed to grow with lactose. In two cases, *viz. Helicoma maritimum* and *Diplodia orae-maris* — both Fungi imperfecti —, the

Table 3. *Effect of thiamin and yeast extract on the growth of three species of marine fungi.*

Species tested	Dry weight of mycelium in mg.		
	No addition	Thiamin 100 µg./l.	Yeast extract 40 mg./l.
Pleospora purpurascens	0.5 ± 0.1	4.2 ± 0.3	9.0 ± 0.6
Trematosphaeria orae-maris	3.7 ± 0.9	110.1 ± 2.3	22.5 ± 0.4
Diplodia orae-maris	68.7 ± 3.4	47.3 ± 2.4	90.4 ± 1.6

Table 4. *Growth of three species of marine fungi with different sugars as carbon sources. 100 µg. thiamin per litre nutrient solution. The asparagine of the standard medium was exchanged for potassium nitrate, 1.7 g. per litre. The sugars were tested in quantities of 10 g. per litre.*

Sugar tested	Dry weight of mycelium in mg.		
	Ceriosporopsis maritima	*Helicoma maritimum*	*Diplodia orae-maris*
Glucose	59.0 ± 3.7	10.3 ± 0.2	3.1 ± 0.8
Fructose	35.3 ± 3.1	15.9 ± 0.3	2.0 ± 0.3
Galactose	46.5 ± 2.5	8.1 ± 0.1	1.4 ± 0.2
Maltose	83.0 ± 2.5	17.4 ± 1.7	2.5 ± 0.1
Sucrose	71.0 ± 2.3	13.1 ± 1.0	1.4 ± 0.1
Lactose	15.4 ± 0.6	22.9 ± 0.7	7.8 ± 2.0

most vigorous growth was obtained with lactose; for most of the other species maltose proved to be the best carbon source of those so far tested.

The investigation is to be continued.

Summary

The nutritional requirements of some marine Ascomycetes (8 species) and Fungi imperfecti (2 species) were studied. Only one species (*Halophiobolus opacus*) required a particular combination of sea water salts. Five species were totally or partially thiamin-heterotrophic. In all cases nitrate could be utilized as a source of nitrogen. Seven species were able to grow with lactose and all ten with galactose.

References

Barghoorn, E. S. & Linder, D. H.: Marine fungi: Their taxonomy and biology. — Farlowia, 1: 395. 1944.

Fries, L.: Studies in the physiology of Coprinus. I. Growth substance, nitrogen and carbon requirements. — Svensk Bot. Tidskr., 49: 475. 1955.

Harder, R. & Uebelmesser, E.: Über marine saprophytische Chytridiales und einige andere Pilze vom Meeresstrand. — Archiv f. Mikrobiol., 22: 87. 1955.

Höhnk, W.: Studien zur Brack- und Seewassermykologie. V. Höhere Pilze des submersen Holzes. — Veröff. d. Instituts f. Meeresforsch. in Bremerhaven, 3: 199. 1955.

Lilly, V. G. & Barnett, H. L.: Physiology of the fungi. — McGraw-Hill Book Co., Inc. 1951.

Vishniac, H. S.: Marine mycology. — Trans. New York Acad. Sci., Ser. II, 17: 352. 1955.

ZoBell, C. E.: Marine microbiology. — Chronica Botanica Co., Waltham, Mass. 1946.

A New Method for the Cultivation of Isolated Roots

By

Miguel Raggio and Nora Raggio

Department of Botany, University of California, Berkeley
(Received May 10, 1956)

The conventional method of isolated root culture under sterile conditions, in which the organ is submerged in standing or shaken liquid media or lies on the surface of an agar medium, has proved very useful in studying the factors which permit growth and development in a number of excised roots (White, 2, and Bonner and Addicott, 1). It has been found that isolated roots generally require in the nutrient medium inorganic salts, including micronutrient elements, an energy and carbon source, and certain growth factors, especially the B vitamins. It is evident however, that in the conventional method the root grows in very unnatural environmental conditions, absorbing directly through its entire surface both the required organic and inorganic nutrients from the medium.

In an attempt to imitate more nearly the natural circumstances of root growth, we have resorted to a separation of the medium (a modification of White's medium (2) containing $FeCl_3 \cdot 6H_2O$ instead of $Fe_2(SO_4)_3$, with added trace elements: 1.5 mg/l $Na_2MoO_4 \cdot 2H_2O$ and 0.04 mg/l $CuSO_4 \cdot 5H_2O$). The organic constituents of the medium are supplied via the root base as in the intact plant and the inorganic salts constitute the medium in contact with the rest of the root.

The physical separation of the two parts has been achieved in two ways: in the first procedure (Figure 1) both media are solid. A 20 ml. volume of a 1.5 per cent agar inorganic medium is poured into an 11 cm. Petri dish. A radial groove is made aseptically in the agar, using a hot Pyrex glass rod. The organic medium (sucrose, glycine, vitamins) is contained in Kimble glass shell vials (12×35 mm.). Each vial is filled with 2.7 ml. of 1 per cent
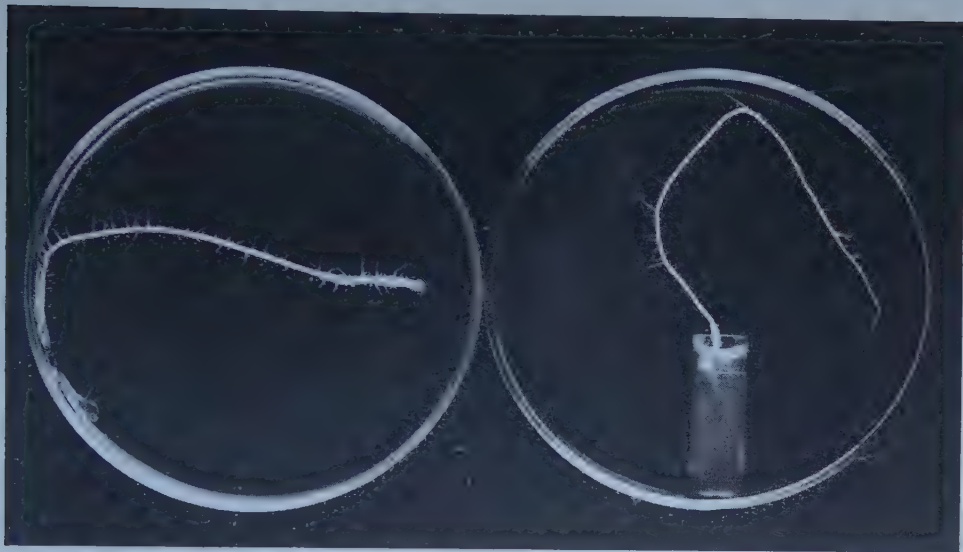
Figure 1. *Growth of bean roots in the conventional* (left), *and new* (right) *methods, after 15 days.*

agar organic medium and is autoclaved at 15 p.s.i. for 15 min. A glass vial is transferred to the groove in each Petri dish (see Figure 1). The basal end (about 5 mm.) of an excised root is next pushed into the organic medium in the vial with forceps. The tip of the root grows into the inorganic medium in the dish. Instead of using agar as the solid substratum in the Petri dish, the inorganic medium can be equally well supplied in sterilized soil, sand or vermiculite.

In the second procedure, the organic medium is solid, the inorganic medium, liquid (Figure 2). The latter is poured (to a height of 115 mm.) into a dry sterilized 20×150 mm. Pyrex test tube capped with an inverted 25×50 mm. glass vial. The organic medium is contained in Kimble glass shell vials (15×45 mm.) each inserted into the lower half of a halved cork perforated with a single hole. The vial with its cork collar fits snugly inside a 25×50 mm. vial, the two vials and the cork thus forming a unit. The inner vial is filled with a 1 per cent agar organic medium and is sterilized by autoclaving. The base of an excised root is pushed into the vial with forceps. The capping vial which covers the mouth of the test tube is then replaced by the inverted two-vial unit, so that the final assemblage is obtained (Figure 2). The level of the inorganic medium may be lowered in the case of roots which do not tolerate submersion; in this case a strip of filter paper of the same width as the tube and about two thirds of its height may be
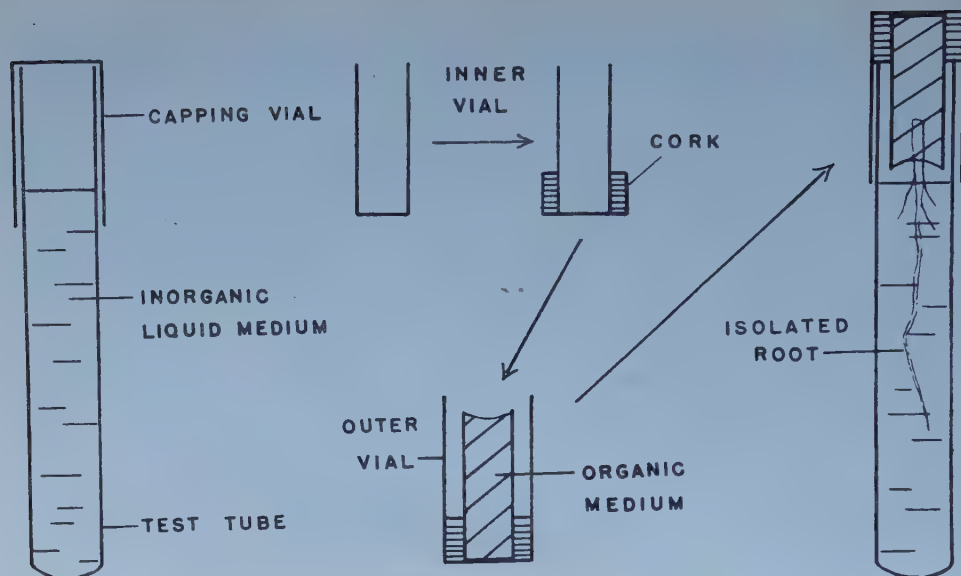
Figure 2. *Illustrat'ng the new method of isolated root culture in which the organic moiety of the medium is supplied in agar and the inorganic moiety, in the liquid form*

used. Also the med:um can be supplied in the solid form (an agar slant, for example) in a way similar to the first procedure described.

Figure 1 shows the results obtained with the conventional and the new method in the gro vth of isolated roots of *Phaseolus vulgaris* Table 1 shows the comparative growth and branching obtained with several methods of excised root cultu e Bean roots grown by any of the variations of the conventional method in modified White's medium showed extensive browning Roots grown by the new method showed no browning. In comparing these results it should be borne in mind that in the new method much smaller absolute amounts of organic nutrients are provided via a much smaller root surface. To compensate for this it may prove useful to increase the concentration of the organic medium in the vial.

The physical separation of the organic and inorganic moieties of the nutrient medium which is rendered possible by this method has been instrumental in obtaining a high percentage of nodulation on isolated bean and soybean roots inoculated with rhizobia. When, following a few earlier attempts by other authors we tried to apply the conventional method of root culture to a study of the nodulation of excised legum nous roots, two main difficulties were encountered: (i) the bacteria grew too abundantly for the welfare of the root, and (ii) the presence of nitrate and sucrose in the medium — which are indispensable for sustained root growth — was already

Table 1. *Comparison of the growth in length and branching of isolated bean roots grown by different methods.*

Both the liquid standing and the liquid shaken cultures were carried out in 50 ml. modified White's medium in 125 ml. Erlenmeyer flasks. Shaking was provided by a horizontal-type mechanical shaker which provided a vigorous swirling motion. The agar cultures were carried out in 20 ml. of a 1.5 per cent agar modified White's medium in 11 cm. Petri dishes. The cultures by the new method were carried out as described in the text All cultures were kept in the dark at 25° C and the measurements were made at the 8th day after excision. Root tips were cut from 25 to 35 mm. primary radicles of black wax bean, *Phaseolus vulgaris,* var. Pencil Pod, germinated for 4 days.

Culture method	Number of roots	Mean length (m.m.) and stand. errors	Mean number of branches and standard errors
5 mm. tips			
Liquid standing	11	25 ± 1	0
Liquid shaken	12	66 ± 2	28 ± 1
Agar	9	49 ± 5	9 ± 2
New method, Petri dish	12	48 ± 5	6 ± 2
20 mm. tips			
Liquid standing	11	40 ± 3	0
Liquid shaken	5	90 ± 3	36 ± 4
New method, test tube	7	96 ± 11	11 ± 3
Agar	9	110 ± 5	45 ± 6
New method. Petri dish	9	89 ± 7	23 ± 5

known (from whole plant studies) to inhibit nodule formation. These obstacles were overcome by using the procedure described above in which inoculation with bacteria is made in a nitrogen-free inorganic medium. This work will be reported at length elsewhere.

The method appears to have many applications in physiological studies of plant development. In the procedure described, the vials substitute for the cotyledons and the shoot system of the plant. Similarly one could attempt to replace the root tip with a vial applied to the decapitated root. Further. the technique should be applicable to a variety o other problems. including the transport of substances, polarity and correlation phenomena and to root physiology in general.

References

1. Bonner, J. & Addicott, F.: Cultivation in vitro of excised pea roots. — Bot. Gaz. 99: 144. 1937.
2. White. P. R.: A handbook of plant tissue culture The Ronald Press Co., New York, N.Y. 1943.

The Entry of Nitrate into Fungal Cells

By

ANNE MacMILLAN

Imperial Chemical Industries Ltd., Akers Research
Laboratories, Welwyn, Herts.
(Received May 25, 1956)

Introduction

In an earlier paper (MacMillan, 1956 a) it was shown that fungal cells are readily permeable to ammonia, which enters the cells and equilibrates mainly as its undissociated molecule. The present study is concerned with the entry of nitrate which is entirely ionic so that its penetration into the cell may be expected to be governed by different factors. *Scopulariopsis brevicaulis*, which has been extensively studied in this laboratory, was used in the present work. The nitrate content of mycelium which had been shaken with nitrate under various conditions was measured. Evidence was obtained that active accumulation of nitrate against a concentration gradient may occur under certain conditions.

In addition, the effect of ammonia on the entry of nitrate was examined since the presence of ammonia in the culture medium (and therefore in the cells) interferes with the assimilation of nitrate in this, and other, fungi.

Experimental Methods

These were generally as described by MacMillan (1956 a) and the same strain of *Scopulariopsis brevicaulis* Sacc. (Bain) was used. It was maintained on Czapek-Dox agar (nitrogen as nitrate) and for experiment was grown on a reciprocal shaker in synthetic medium containing dextrose and potassium nitrate. An even suspension of mycelium was harvested after 3 days' growth, and the mycelium washed 2 or 3

times with distilled water before use. For most experiments the mycelium was suspended in solutions of potassium nitrate in phosphate buffer (pH 7) made from mixtures of $KH_2POH + Na_2HPOH$) and shaken for aeration during the experimental period.

Measurement of Nitrate assimilation was made as described by Morton and Mac-Millan (1954). The loss of nitrate from the solution per unit weight of mycelium was determined, as well as the unassimilated nitrate present in the mycelium.

Extraction of mycelium for determination of nitrate content. A 1—3 g. sample of mycelium was dried in the mangle as described below, without washing off the nitrate solution, weighed quickly and heated in approximately 10 times its weight of phosphate buffer (pH 7) in a boiling water bath for 3 min. The cooled extract was made to volume and nitrate determined on aliquots of the whole suspension and calculated as mg. N/g. fresh weight (F.Wt.) as indicated below. Replicates agreed to within 10 per cent.

Fresh weight of mycelium. The mycelial suspension was filtered rapidly on a Buchner funnel at the water pump and the mycelium passed 4 times through a small mangle with 6 filter papers on each side before weighing. Fresh weight/dry weight ratios were reproducible to ± 3 per cent in this way.

Estimation of the liquid adhering to the mycelium. To overcome the difficulty of separating the mycelium completely from the nitrate solution in which it is suspended the procedure of MacMillan (1956 a) was adopted and 40 per cent of the fresh weight of the mycelium after "mangling", was taken to represent the adhering solution. The mycelium therefore was not washed free from the nitrate solution and a correction was made for 0.4 ml. external nitrate solution (whose concentration was determined) per gram fresh weight of mycelium. Thus, NO_3 in mycelial extract/g. total F.Wt. $= NO_3$ in 0.6 g. F.Wt. mycelium $+ NO_3$ in 0.4 ml. external solution.

Pressing procedure for cell sap. The mycelium was pressed at approximately 350 lb./sq. in. after rapid ether treatment (Crowdy and Pramer, 1953). The liquid obtained consists of cell fluid together with external solution adhering to the mycelium.

Nitrate estimation. In mycelial extracts and the cell sap the nitrate (10—80 µg. N) was estimated after reduction to ammonia. 4 ml. extract was left to stand overnight with 0.6 ml. 50 per cent v/v H_2SO_4 and 250 mg. reduced iron powder. The ammonia was liberated *in vacuo* with 5 ml. 3 N KOH and collected in dilute HCl and Ness-lerised (Koch and Hanke, 1948). Ammonia blanks without iron were run simultaneously. Recovery of nitrate added to the killed suspension was 98—99 per cent by this method.

In the solutions in which the mycelium was shaken, larger amounts of nitrate were estimated by the method of Conway and Byrne, (1933) using 50 mg. Devardas alloy and 0.5 N KOH to reduce the nitrate and boric acid as absorbent for ammonia. The presence of nitrite was always checked with α-naphthylamine but was never found in more than trace amounts.

Results

In all experiments the mycelium of *S. brevicaulis* was harvested a little less than 3 days after inoculation, when the nitrate of the culture medium
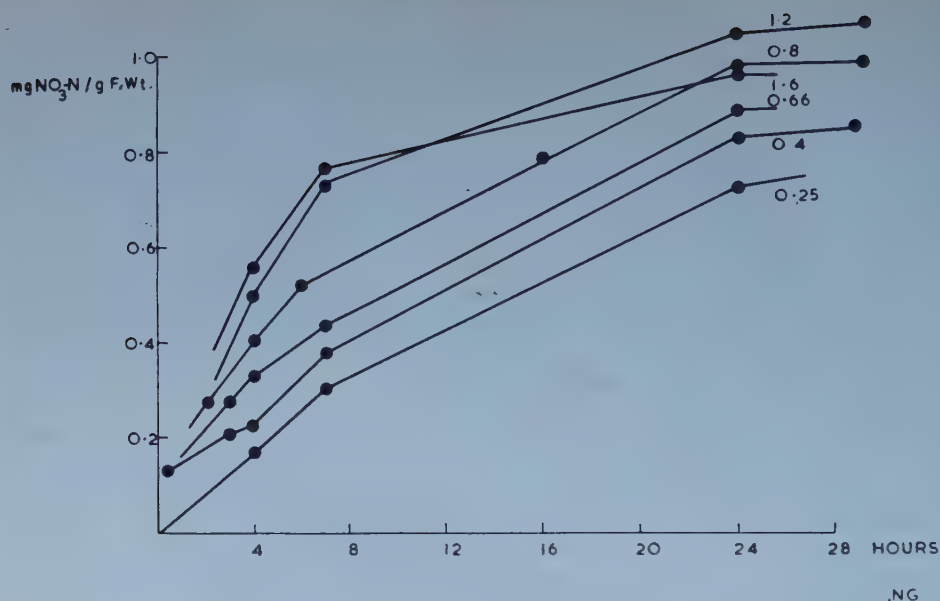
Figure 1. *Nitrate content of mycelium of S. brevicaulis after shaking in phosphate buffer with KNO₃ at concentration shown* (as mg. N/ml.).

is just exhausted and the mycelium contains no free nitrate. If this mycelium is shaken in phosphate buffer (pH 7) containing potassium nitrate the nitrate content of the mycelium rises for about 24 hr. until a steady value is reached (figure 1). The internal level of nitrate lies between 0.7 and 1.0 mg. NO_3-N/g. F.Wt. Thus the final level of nitrate in the mycelium varies much less than the external concentration in the range covered by these experiments, and appears to reach a maximum value which does not rise with the external concentration. At the higher concentrations the overall nitrate level in the cells is similar to the external concentration, but at the lower external concentrations the internal nitrate concentration represents a considerable accumulation in the cells.

The rate of entry of nitrate is more rapid during the first few hours and during this initial period it is dependent to some extent on the external concentration. Subsequently, however, the rate of entry appears to be the same at all concentrations regardless of whether accumulation occurs.

The data in figure 1 are from two separate experiments. Similar results were obtained in a number of others, although some variation between experiments was observed in the final levels of nitrate reached. This must be due to variability in the fungus, since replicates in the same experiment agreed to within 10 per cent, usually much less.
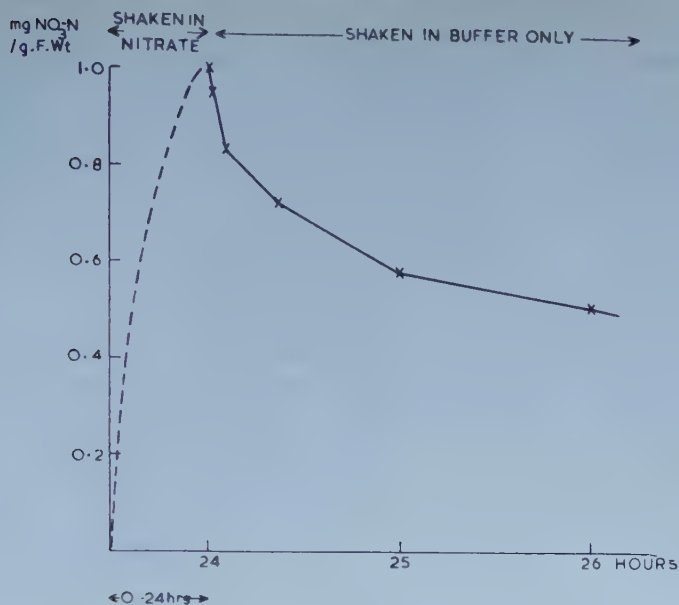
Figure 2. *Effect of washing in buffer on nitrate content of mycelium previously shaken in buffer containing KNO₃ (0.8 mg. N/ml.).*

The values for the concentration of nitrate in the mycelium are corrected for the nitrate solution which adheres to the surface. They are, therefore, a measure of the nitrate actually in, or adsorbed on, the cells. The validity of the method for correcting for the adhering nitrate solution is confirmed when mycelium, previously shaken 24 hr. in nitrate solution, is washed free from the external nitrate before it is extracted. After rapid washing with buffer, the nitrate concentration in the mycelium, when corrected for nitrate-free surface liquid, is only slightly less than in the unwashed control (figure 2). More prolonged washing, however, shows that nitrate is lost quite rapidly from the cells, and after 1 hour's washing has dropped from 1.0 mg. to 0.6 mg. N/g. F.Wt. Subsequently the outward movement of nitrate is much slower.

If the cell sap is expressed from mycelium which has been shaken in nitrate solutions for 24 hours, it is found to contain nitrate. The concentration in the expressed sap is very similar to the overall concentration in the mycelium as determined by the extraction method (Table 1) so that it appears that much of the nitrate in the cells is in solution in the cells. This method does not give a quantitative measure of the internal concentration since the sap expressed from the cells is mixed with external solution adhering to the cells, and the mycelium is not washed free from external nitrate. Nevertheless,

Table 1. *Nitrate nitrogen in expressed cell sap from mycelium of S. brevicaulis shaken in phosphate buffer (pH 7) containing KNO₃.*

External NO₃ concn. (mg. N/ml.)	Time	mg. NO_3-N/g. F.Wt.	mg. NO_3-N/ml. sap
0.8	Initial	0.01	0
	3 hr.	0.31	0.08
	24 hr.	1.07	1.04
0.25	3 hr.	0.20	0.03
	24 hr.	0.73	0.57

Table 2. *Nitrate in mycelium shaken with NO_3^- (0.7 mg. N/ml.) in phosphate buffer or Tris at pH 7.*

Time	NO_3-N/g. F.Wt. (mg.)	
	Phosphate	Tris
2 hr.	0.27	0.29
4 hr.	0.27	0.38
24 hr.	0.73	0.69

Table 3. *Effect of pH on nitrate content of mycelium of S. brevicaulis shaken in phosphate buffer with KNO₃ (0.8 mg. N/ml.).*

Time	pH 5.0	pH 6.9	pH 8
3 hr..........	0.30	0.20	—
6 hr..........	0.56	0.50	—
24 hr..........	1.15	0.97	0.72
29 hr..........	1.05	0.98	—

it is clear that after 24 hr. the concentration of nitrate in the sap is as high or higher than in the external solution. On the other hand, the cell sap from mycelium shaken only 3—5 hours in nitrate solution contains very little nitrate (Table 1). It is possible, therefore, that during these first hours much of the nitrate entering the cells is held by adsorption, the concentration in the cell solution rising only after the adsorptive centres become saturated.

In these experiments the mycelium was shaken in solutions of potassium nitrate in phosphate buffer. The effect of the phosphate and sodium and potassium ions present in the solution on the entry and accumulation of nitrate was therefore examined, since the might affect both the entry and adsorption of nitrate ions.

The mycelium was carefully washed free from the culture medium and shaken in a solution of 0.06 *M* Trishydroxymethylaminomethane and 0.06 *M*
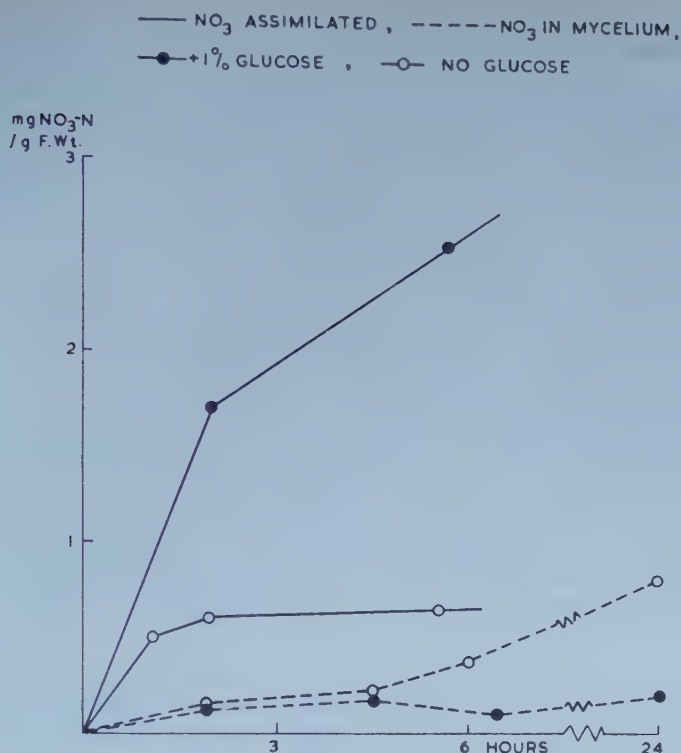
Figure 3. *Comparison of nitrate assimilated and nitrate present in mycelium after shaking in solutions of KNO₃ (0.4 mg. N/ml.) with and without glucose (1 per cent w/v).*

HNO_3 (at pH 7). This solution contains no ions other than H^+ and NO_3^-. Table 2 shows that the nitrate content of cells shaken in this solution is the same as in those shaken in phosphate buffer.

The pH of the nitrate solution does affect the nitrate level in the cells, as is seen in Table 3. Slightly more nitrate is held in the cells at lower pH values, so that between pH 5 and pH 8 the nitrate content after the equilibrium level is reached, falls from 1.1 to 0.7 mg. N/g. F.Wt.

In a study of the entry and accumulation of nitrate in the cells, the question of its removal from the cells by assimilation must be considered. In the experiments described so far, no external energy source was supplied to the mycelium so that assimilation of nitrate to amino acids and proteins stops after 1—2 hours, (MacMillan, 1956 b). Thus for most of the period during which the nitrate content of the cells rises no assimilation occurs. On the other hand, if glucose is supplied in the solution, nitrate is assimilated for many hours, this being accompanied by an increase in dry weight. At
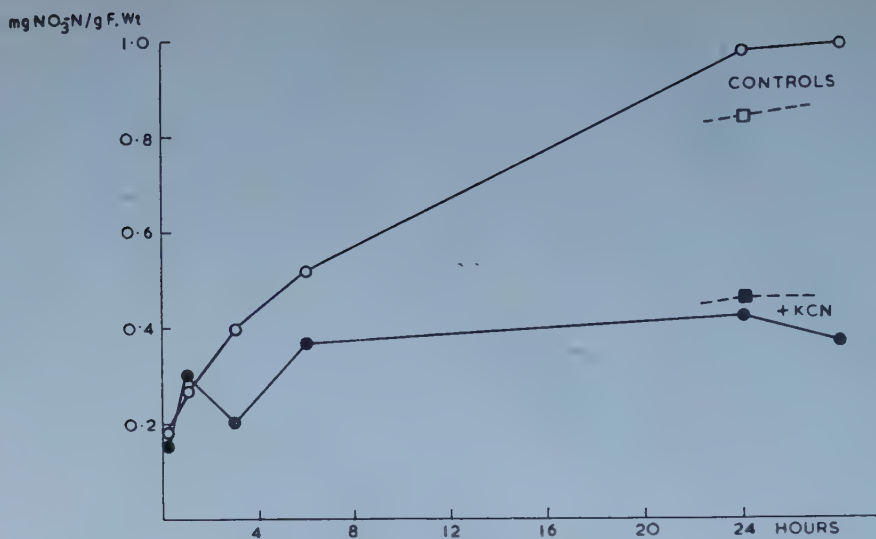
Figure 4. *Effect of potassium cyanide* (0.01 *M*) *on nitrate content of S. brevicaulis shaken in phosphate buffer containing KNO₃.* (——— 0.8 mg. NO₃-N/ml., - - - - - 0.4 mg. NO₃-N/ml.).

the same time, nitrate does not accumulate, the concentration in the cells remaining below about 0.3 mg. N/g. F.Wt. (figure 3). There is likewise no accumulation in the cells during the normal growth of *S. brevicaulis* in the nitrate culture medium; for example, when the medium contained 0.53 mg. N/ml. the cells contained 0.10 mg. N/g. F.Wt.

Thus when nitrate assimilation occurs, nitrate is removed from the cells by assimilation as rapidly as it enters. The rate of entry moreover is much greater than under conditions in which assimilation is absent and nitrate accumulates. In the absence of glucose it takes about 24 hr. for 1 mg. N/g. F.Wt. to be accumulated, while during assimilation this amount of nitrate is taken up in about 2 hr.

The role of respiratory energy in the entry and accumulation of nitrate was next examined. When nitrate is supplied to nitrate-free mycelium in phosphate buffer, so that nitrate enters the cells, there is usually no rise in the endogenous respiration rate although a slight temporary rise may be observed (MacMillan, 1956 b). A small amount of nitrate is converted to amino acids for 1—2 hr. in the absence of glucose so that any extra respiration is probably due to such assimilation, which requires energy, and not to the entry of nitrate ions into the cells. Support for this comes from the fact that addition of ammonia which has been shown to enter the cells passively produces a similar rise in endogenous respiration (MacMillan, 1956 a).
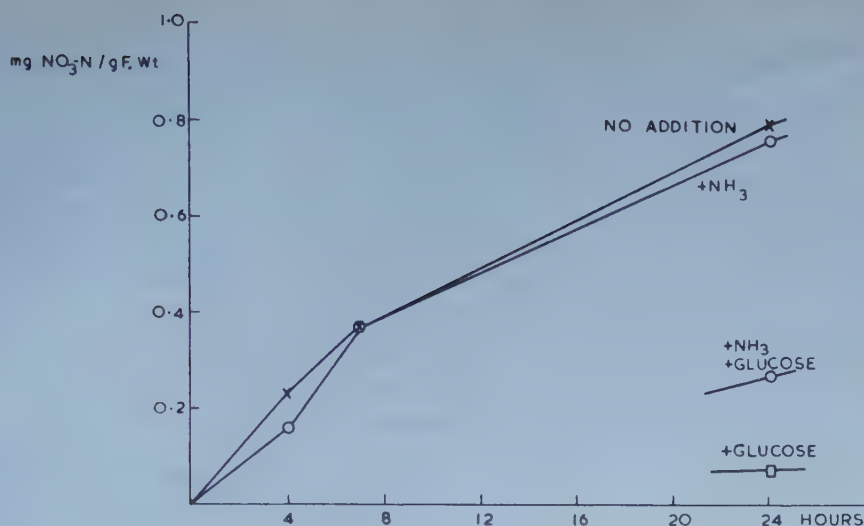
Figure 5. *Effect of ammonium sulphate* (0.4 mg N/ml.) *and glucose* (1 per cent) *on nitrate content of mycelium of S. brevicaulis shaken in phosphate buffer containing KNO₃* (0.4 mg. N/ml.).

If glucose is supplied together with nitrate so that there is a greater rate of nitrate entry, the respiration rate is doubled. Some of this increase may be connected with the entry of nitrate ions. A large part, if not all, of the extra respiration, however, is due to the continued assimilation. This is apparent, again by analogy with ammonia assimilation, which results in an even greater rise in respiration rate (MacMillan, 1956 b).

There is here no clear evidence that the entry or accumulation of nitrate into the cells is connected with an increase in respiration rate. On the other hand, results with inhibitors have shown that respiratory energy is requierd for nitrate accumulation. Fig. 4 shows the effect of 0.01 M potassium cyanide on the nitrate level in the cells in the absence of glucose. The concentration of cyanide used inhibits about 70 per cent of the endogenous respiration. During the first few hours cyanide has little effect on the nitrate level in the cells, so that about 0.4 mg. N/g. F.Wt. can be held in the cells even when respiration is largely inhibited. Subsequently, cyanide clearly inhibits the continued rise in nitrate content to levels above the external concentration. Very similar results were obtained with 0.02 M sodium azide.

It was of interest to examine the effect of ammonia on the entry of nitrate since ammonia specifically inhibits the assimilation of nitrate (Morton and MacMillan, 1954). Figure 5 shows that the accumulation of nitrate in the absence of glucose is not affected by ammonia (as 0.03 M $(NH_4)_2SO_4$). How-

ever, in the absence of glucose, assimilation of ammonia stops after 1—2 hr. and there is evidence that ammonia exerts its inhibitory effect only when it is being assimilated. Thus when glucose is supplied, ammonia assimilation is prolonged but nitrate assimilation is inhibited. It is seen in figure 5 that under these conditions although the nitrate level in the cells is higher than during nitrate assimilation (i.e. without ammonia), it is much lower than when, in the absence of glucose, neither nitrate nor ammonia are assimilated. This may be connected with the large increase in dry weight which occurred in the 24 hr. (about 120 per cent over the initial).

Discussion

The experiments described have shown that nitrate can be accumulated against a concentration gradient by cells of the fungus *S. brevicaulis*. When shaken in nitrate solutions the nitrate content of the mycelium rises for about 24 hr. and reaches a level which is only slightly affected by the external concentration. Accumulation of nitrate therefore occurs when the external concentration is below this level (about 0.07 *M*).

During the period of nitrate accumulation two phases can be distinguished. The first, lasting 3—6 hr., appears to be a passive process, not inhibited by cyanide. During this phase the rate of entry of nitrate, and therefore the nitrate level in the cells, depends on the external concentration, but no accumulation occurs. Most of the nitrate appears to be held by adsorption in the cells since little is found in solution in the expressed cell sap. During the second phase, accumulation of nitrate occurs, and this is inhibited by cyanide and azide. The rate of entry is slower and is independent of the external nitrate concentration, indicating that it is metabolically controlled, probably linked with the respiration rate.

While virtually nothing is known of ion entry into fungal cells there is evidence that, in other types of cell, the initial entry of ions involves a passive equilibration of part of the cell volume with the external concentration. This varies from 13 per cent of the volume of root cells (Hope and Stevens, 1952) to 35 per cent in *Staphylococcus* cells (Britten, 1953) and 75 per cent in *E. coli* cells (Cowie and Roberts, 1954). The initial phase of entry in *S. brevicaulis* is probably concerned with such a "non-metabolic" region, although from present results an estimate of its volume cannot be made. Moreover, if adsorption occurs this will affect its apparent volume.

There is much evidence that adsorption or ion-exchange plays a vital role in the initial entry of ions into higher plant cells (Lundegårdh, 1955), and in this respect the process in *S. brevicaulis* is apparently analogous. The

subsequent metabolic accumulation against a concentration gradient in higher plants probably involves active transport of ions from the cytoplasm to the vacuole. It is not known whether this is the case in fungal cells, but the hyphae of *S. brevicaulis* contain numerous vacuoles.

Active transport in higher plant cells is thought to be mediated either by carrier molecules or directly by the cytochrome oxidase system. Both these modes of accumulation are dependent on respiration rate and from the present evidence either could be the one operating in fungal cells. No clear evidence was obtained of an increase in respiration rate accompanying the accumulation of nitrate. Such a "salt respiration" usually, though not always, accompanies ion accumulation in higher plants, and is due to the activity of the cytochrome system. Indeed, in the present work, cyanide and azide, known inhibitors of cytochrome oxidase, inhibit both nitrate accumulation and respiration. However, the occurrence of cytochrome oxidase in *S. brevicaulis* has not been demonstrated but it is known to occur in a number of fungi (Darby and Goddard, 1950; Boulter, 1955).

If glucose is supplied together with nitrate the respiration rate in *S. brevicaulis* is increased. Under these conditions the rate at which nitrate is taken up is increased about ten-fold but the nitrate entering the cells is assimilated as rapidly as it enters, and no accumulation occurs. Therefore, the rate at which nitrate accumulates in the absence of glucose is not the maximum rate at which it can enter the cells. The slower rate of entry in the absence of glucose may be due simply to the slower rate of respiration. On the other hand it may be that the rate of entry is actually the same in both cases but that if nitrate is not continuously removed by assimilation, the entry is accompanied by a significant leakage from the cell. (A rapid outward movement of nitrate was shown to be possible). This would reduce the rate of accumulation and the final steady level of nitrate in the cells would represent an equilibrium between the rates of movement of nitrate in both directions.

The absence of accumulation when assimilation occurs may be due to a limiting rate of entry. It may also be due to a diversion of metabolic energy to the assimilatory processes, so that energy is not available for the maintenance of a high nitrate level in the cells. This is supported by the finding that if ammonia is present together with nitrate, the assimilation of ammonia inhibits nitrate assimilation. Thus, although the removal of nitrate from the cells by assimilation is prevented there is still no accumulation. The presence of ammonia without assimilation (i.e. without glucose) has no effect on nitrate accumulation. Therefore it seems that it is the process of nitrogen assimilation and its energy requirements which prevent a simultaneous accumulation of nitrate. Since the accumulation of nitrate does not occur during nitrogen assimilation this process cannot be a significant factor in

the growth of *S brevicaulis* in carbohydrate-rich media. This is in contrast with the finding that in higher plants ion accumulation is frequently associated with active metabolism and protein synthesis (Steward and Street. 1947), although this is not always the case (Humphries, 1952, Lundegårdh, 1955). Nevertheless. the process of nitrate entry into the fungal cells appears to be essentially similar to that in higher plants.

Summary

When *Scopulariopsis brevicaulis* is supplied with nitrate, the level of nitrate in the cells rises steadily for about 24 hr. until an equilibrium level between 0.07 and 0.10 M NO_3^- is reached in the cells. Thus when the external concentration is less than this, nitrate is accumulated against a concentration gradient. Evidence was obtained for an initial passive entry of nitrate, followed by an active process of accumulation which is inhibited by cyanide and azide.

If glucose is supplied together with nitrate, entry of nitrate into the cells is more rapid but no accumulation occurs nitrate being assimilated as rapidly as it enters. The results suggest that, although the respiration rate is increased, under these conditions metabolic energy is not available to support simultaneous accumulation of nitrate.

References

Boulter, D.: The oxidase of Polystictus versicolor and Neurospora crassa in relation to their endogenous respiration. — Biochem. J. 60: xxi. 1955.
Britten, R.: After Gale, E. F. (1954). — Soc. Exp. Biol. Symp. 8: 212. 1953.
Conway, E. J. & Byrne, A.: An absorption apparatus for the micro-determination of ammonia. — Biochem. J. 27: 419. 1933.
Cowie, D. B. & Roberts, R. B.: The permeability of microorganisms to inorganic ions, amino acids and peptides. — J. cell. & Comp. Physiol. 44: 327. 1954.
Crowdy, S. H. & Pramer, D.: The occurrence of translocated antibiotics in expressed cell sap. — Ann. Bot. N. S. 19: 79. 1955.
Darby, R. T. & Goddard, D. R.: The effects of cytochrome oxidase inhibitors on the cytochrome oxidase and respiration of the fungus Myrothecium verrucaria. — Physiol. Plant. 3: 435. 1950.
Hope. A. B. & Stevens, P. G.: Electric potential differences in bean roots and their relation to salt uptake. — Austr. J. Sci. Res. B. 5: 335. 1952.
Humphries, E. C.: The absorption of ions by excised root systems — J. Exp. Bot. 3: 291. 1952.
Koch, F. C. & Hanke, M. E.: Practical Methods in Biochemistry. — Williams & Wilkins Co., Baltimore. 1948.

Lundegård, H : Mechanisms of absorption, transport, accumulation, and secretion of ions. — Ann. Rev. Plant Physiol. 6: 1. 1955

MacMillan, Anne: The entry of ammonia into fungal cells. — J. Exp. Bot. 7: 113. 1956 a.

— The relation between nitrogen assimilation and respiration in Scopulariopsis brevicaulis. — Physiol Plant 1956 b. In press.

Morton, A. G. & MacMillan, Anne: The assimilation of nitrogen from ammonium salts and nitrate by fungi. — J Exp Bot. 5: 232. 1954

Robbie, W. A.: Use of cyanide in tissue respiration studies. — Methods in Medical Research 1: 307. 1948.

Steward, F. C. & Street, H. E · The nitrogenous constituents of plants. — Ann. Rev. Biochem. 16: 471. 1947.

Light-Induced Stomatal Transpiration of Etiolated Wheat Leaves as Related to Chlorophyll *a* Content

By

Hemming I. Virgin

Botanical Laboratory, Lund
(Received July 20, 1956)

Introduction

The importance of chlorophyll pigments for the light-induced stomatal movements has been discussed by several authors. The opinions of the rôle of the pigments in this connection differ considerably. Lloyd (10) found evidence that guard cells with chlorophyll-free plastids were capable of movement in spite of their lack of chlorophyll. Scarth (14), on the other hand, found no immediate effect of light on guard cells located above chlorophyll-free tissue. He noted, however, that the tissue responded gradually with time and thought that the response spread from the cells located next to the chlorenchyma. Also Harms (6) obtained the result that light-induced opening movements of guard cells, situated over chlorophyll-free tissue, were proportional to the intensity of light absorbed by the tissue. In a short report Wilson (25) states that stomata of non-chlorophylleous Coleus blumei responded to light. The conclusion that the plants were free from chlorophyll was drawn from the fact that the guard cells showed no fluorescence. No quantitative chlorophyll determination was performed. Also dark-grown plants of sweet potato showed movements of their guard cells when placed under a light of approximately 1,000 foot candles for 10 minutes. After half an hour many guard cells had opened quite widely, and still no fluorescence was observed in the plastids of any of the cells of the leaf. From this the conclusion was drawn "that guard cells containing non-fluorescing plastids and located over chlorophyll-free tissue are capable of exhibiting light-induced

movements". In the literature concerning stomatal mechanism, however, reports can also be found of stomata which show no light-response at all when chlorophyll is lacking. Paetz (11) found no light-induced stomatal movements whatsoever in chlorophyll-free parts of variegated leaves of Caladium. On the other hand, he was able to show that a change in the humidity of the air could cause changes in the aperture of the stomata very similar to those caused by light. Also Liebig (9) showed that only light which was absorbed by green plastids in the guard cells produced stomatal movements. It may thus be possible that at least some of the stomatal movements reported in chlorophyll-free stomatas can be referred to changes in the water content under the influence of the heat caused by the irradiation.

The findings of the present investigation support the idea that a real correlation exists between the chlorophyll content of the tissue and the ability of the stomata to respond to light. The experiments were performed with the corona-hygrometer developed by Andersson and Hertz (2). It was shown in three earlier papers (Andersson *et al.*, 3; Rufelt, 13; Virgin, 24) that this instrument can be used for the determination of stomatal transpiration. It is possible by means of this apparatus to determine accurately very small changes in the rate of transpiration.

Plant Material and Methods

The plant material used for this study consisted of etiolated dark-grown leaves of wheat (Weibull's orig. "Eroica"). The seedlings were grown in complete darkness in a thermostat-regulated darkroom at 22° C until used for the experiments. A detailed description is given in Virgin (24). When the plants were taken out of the room they were illuminated for only a fraction of a minute with very dim green light, which is incapable of transforming the protochlorophyll to chlorophyll a (Koski *et al.*, 8). The plants were used when the leaves had reached a length of circa 12 cm. (6—7 days after the planting).

The determination of transpiration was performed in the same way as described in an earlier paper (Virgin, 24). During the measurements the plants were intact and their roots immersed in an aerated nutrient solution. The arrangements for the illumination were also the same as previously described.

The estimation of the chlorophyll a content of the illuminated leaves was made spectrophotometrically according to the method of Koski *et al.* (8). Five leaves with an average fresh weight of 0.2 g were ground with sand and 25 ml of acetone in a mortar. 25 ml of ether was added. The acetone-ether extract was then washed with water 3 times in a separatory funnel and finally run into a graduated cylinder. The ether solution was made to measure exactly 15.0 ml. For further details; see Koski *et al* (8). The optical density of the solutions was measured by means of a Bausch and Lomb grating spectrophotometer, Model: Spectronic 20, at the wave length 662 mμ. At this wave-length ether solutions of chlorophyll a possess an absorption maximum. In the

calculation of the concentration of chlorophyll a, 100.9 was used as the value for the specific absorption coefficient (Smith and Benitez, 18).

Experimental

Transpiration Measurements

The object of this investigation was to find out whether any relationship exists between the chlorophyll a content of the plant and the ability of the stomata to respond to light. The experiments were arranged in the following way. For each experimental series 10 wheat plants were placed in the transpiration chamber and the measurement of the transpiration was performed. The transpiration in darkness was followed for at least 30 minutes. The increase in the relative humidity of the outstreaming air above that of the incoming air amounted to an average of 2 per cent under the present experimental conditions (Virgin, 24). The small increase in the humidity was due to the cuticular transpiration. After the period in darkness the plants were illuminated with light of different intensities (0.8, 2, 10, 100, 1,000, 10,000, 50,000, and 64,000 meter candles). After varying periods of illumination (1, 2, 3, 4, 5, and 6 hours) the light intensity was increased to 64,000 meter candles which was administered for about half an hour. The results are given in Figure 1. Each series of illumination in the Figure is thus a combination of 6 single experiments. For example, plants have thus been illuminated for 1 hour with an intensity of 0.8 meter candles, whereafter the light intensity was increased to 64,000 meter candles and the transpiration measured for a further period of about half an hour. Fresh material was then placed in the chamber and the measurement of the transpiration was repeated as before with a different period of illumination.

It is evident from Figure 1 that in a completely etiolated plant the stomata do not respond to light — at least not with any change in the aperture, as no change in the transpiration is obtained during the first 2—3 hours of illumination. If the plant is illuminated with as weak light as 0.8 meter candles, the illumination must last at least for 6 hours before any visible effect can be obtained by increasing the light intensity. A constant illumination of the plant for 6 hours does not change the rate of transpiration, which means that this intensity is not high enough within this time to cause a movement. If the light intensity of the constant illumination is increased to 2 meter candles, the first response to 64,000 meter candles of intensity comes after 3 hours of illumination. One can also notice here that there is a very small but noticeable increase in the transpiration during the constant illumination with 2 meter candles. This increase becomes more evident when the
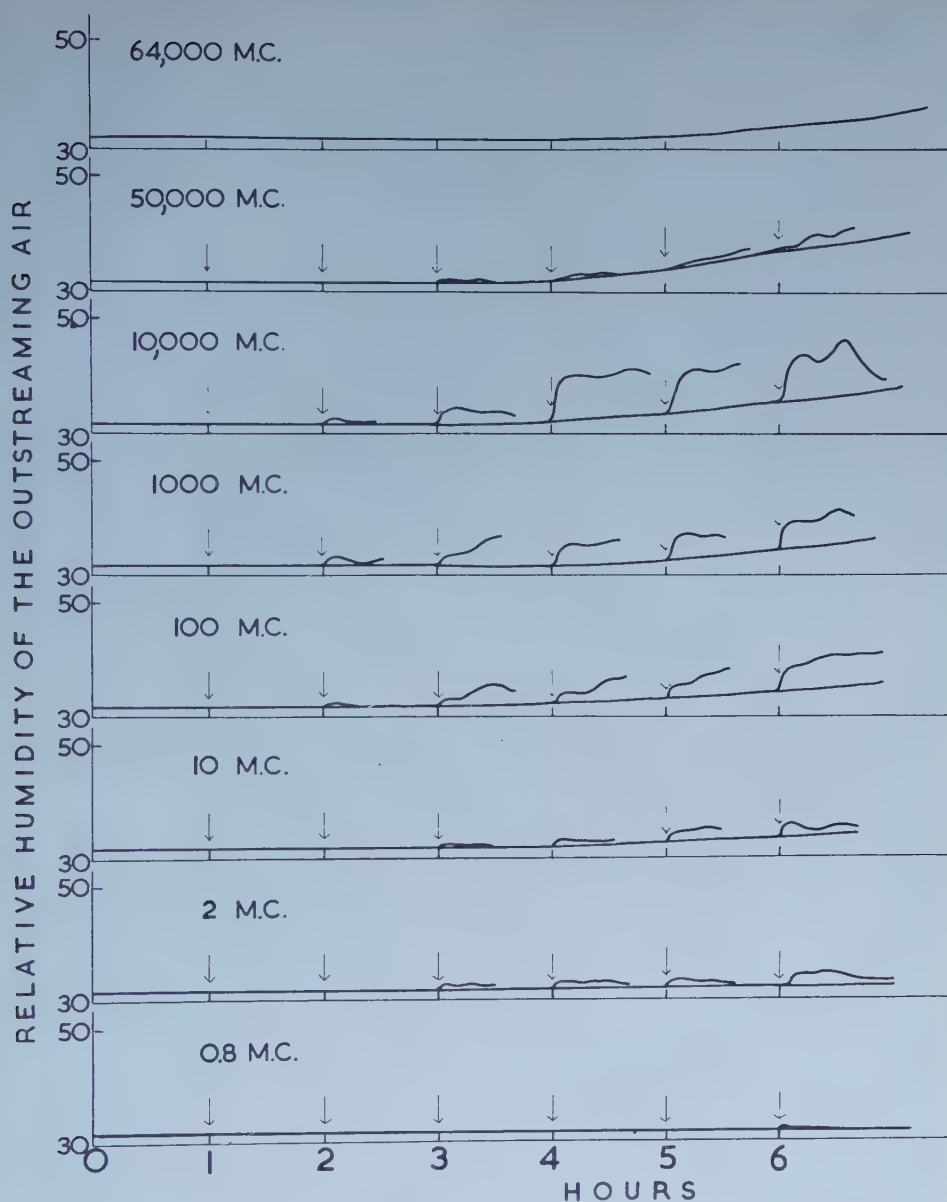
Figure 1. *The transpiration from etiolated wheat leaves under light of different intensities.* Each series represents a combination of 7 single experiments, except for the series with 64,000 meter candles which represents one experiments. At the arrows the light has been increased from the original intensity, shown in figures, to 64,000 meter candles which was given for about half an hour.

light intensity is increased to 10 meter candles. The increase begins after about 3 hours of constant illumination. It can be seen that the increase in transpiration during the continuous illumination of different light intensities never sets in before about 2—3 hours of illumination. It is also apparent that there is a certain light intensity around 10,000—50,000 meter candles, which gives the highest transpiration values. A further increase in the intensity results in lower transpiration values.

If a light intensity of 64,000 meter candles is applied at different intervals from the onset of irradiation with weaker light, it is thus evident that one will never obtain an increase in the transpiration until about 2—3 hours after the onset of the illumination, no matter how strong the intensity of light. As a matter of fact, for high intensities of continuous illumination, *e.g.*, 50,000 meter candles, the response for 64,000 meter candles comes first after 3 hours of illumination. It is quite natural that the response to the stronger light will become less and less the smaller the difference between the intensity of the continuous light and the added light. Disregarding this fact, however, it is obvious from the experimental results that the response to very strong light is smaller than the response to medium light intensities. (In the Figure, the response to 64,000 meter candles of continuous illumination is smaller than to 50,000 and 10,000 meter candles.)

Pigment Formation

The chlorophyll a content of the plant after different periods of illumination is seen in Figure 2. The measurements of the chlorophyll a content of the leaves were performed on other material than that used for the transpiration experiments. This was necessary because of the fact that the termination of the experiments with the illumination with strong light (64,000 meter candles) caused a change in the chlorophyll content. The plants used for the chlorophyll estimation, however, were treated in exactly the same way as those in the illumination series.

The formation of chlorophyll a in wheat leaves seems to follow the same pattern as has been shown valid for barley (Virgin, 23) and for oat (Blaauw-Jansen *et al.*, 4). During a period of about 2—3 hours the formation is very slow. On further illumination an acceleration in the formation sets in which is very obvious, especially in medium light intensities. The "zero" value for the chlorophyll lies at $7.2 \cdot 10^{-3}$ mg per g of fresh leaves. This is the value which will be obtained if the determination of chlorophyll is made on etiolated leaves immediately after they have received a very short light impulse. If chlorophyll a is determined on etiolated leaves and the extraction is made in complete darkness, the value will be near zero. Even the slightest illumina-
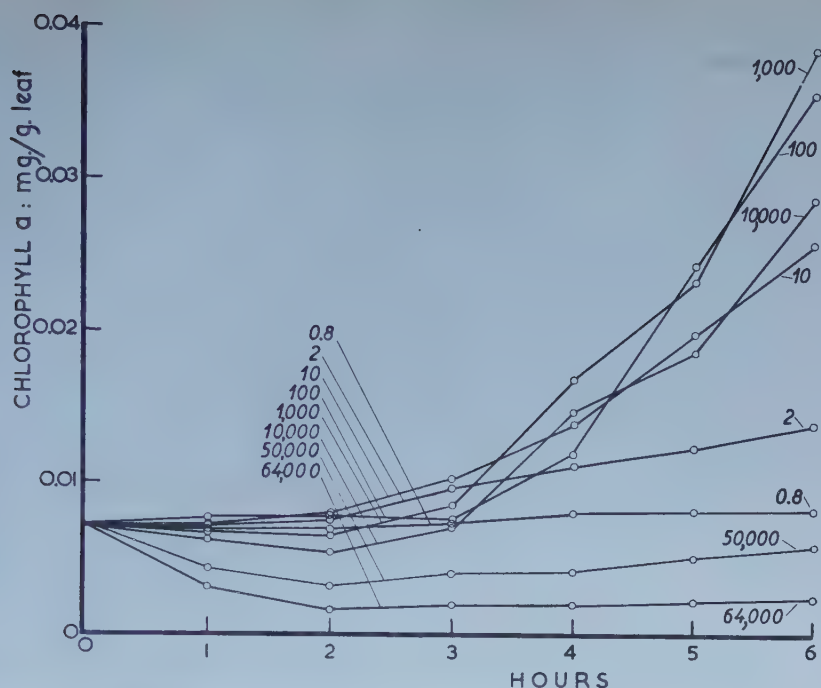
Figure 2. *The chlorophyll a content in etiolated and illuminated wheat leaves.* The chloro-phyll content at zero time represents the chlorophyll immediately formed from the proto-chlorophyll present in darkness (cf. Virgin, 22, 23). The figures denote the light intensity in meter candles.

tion will produce this amount of chlorophyll a from the small amounts of proto-chlorophyll, always present in etiolated leaves (Koski *et al.*, 8; Smith and Young, 19; Virgin, 22, 23). In Figure 2 it can be seen that the further formation of chlorophyll a is dependent on the light intensity. Intensities be-low about 0.8 meter candles do not appreciably increase the chlorophyll a content — at least not during 6 hours' illumination. By increasing the light intensity the chlorophyll formation is accelerated. It is to be noted, however, that this increase sets in first after 2—3 hours. A further increase of the inten-sity causes more chlorophyll to be formed, but only to a certain extent. If the light intensity is increased above around 1,000 meter candles, less and less chlorophyll is formed with increasing intensity. The explanation for this is to be found when studying the conditions in very strong light. One can see in Figure 2 that chlorophyll, already formed, gradually disappears the more rapidly the higher the light intensity. This destruction of chlorophyll has earlier been observed by among others Koski (7) in a study of the trans-formation of protochlorophyll to chlorophyll a. It is particularly obvious

when the transformation takes place at low temperatures where no further chlorophyll is formed after the initial transformation (Smith, 16). In Figure 2 it is shown that at an intensity of 64,000 meter candles some of the firstly formed chlorophyll is destroyed and nothing more — or very small amounts are formed. This means that at the beginning of the illumination with high intensities of light the destruction of pigment exceeds the formation. This also indicates that parts of the mechanism producing protochlorophyll are affected by high light intensities and probably partly destroyed.

In an earlier paper (Virgin, 23) the chlorophyll content in barley after irradiation was determined. The experiments were designed so that the amounts of protochlorophyll were supposed to remain constant under the experimental conditions. The results reported the variation of chlorophyll a using the protochlorophyll concentration as a standard. The resulting calculations showed a remarkable acceleration of the chlorophyll a formation after 2 hours of illumination with 600 foot candles. At the same time the production of pigment during the first 2 hours was smaller than for lower light intensities. These high values for the production of chlorophyll at this intensity should therefore be considered a mis-representation. As the concentration of protochlorophyll has probably decreased under the influence of the strong light intensity, the real values for chlorophyll a are supposed to be considerably lower and probably of the same order of magnitude as for the other medium light intensities.

Conclusions

If the formation of chlorophyll in the guard cells occurs in the same way as in the mesophyll cell layers, the following conclusions can be drawn from the present experimental data as to light-induced stomatal response and chlorophyll content:

In etiolated wheat leaves the stomata do not respond to light unless the content of chlorophyll a in the plant has reached a certain level. At least the stomata do not respond measurably to light during the first 2 hours of illumination when the content of chlorophyll is kept at a low level, regardless of the intensity of light. The ability to respond to light increases with the formation of chlorophyll. If the plant has been illuminated for some hours with a certain light intensity and then is transferred to stronger light, the response to the latter is strongest if the plant has been pretreated with medium light intensities. If an etiolated plant has been subjected to strong intensities of light for several hours, a decrease in stomatal response can be shown which keeps pace with the simultaneously occuring chlorophyll destruction. In the interpretation of the response to different light intensities consideration must also be paid to the photosynthetic activity in different light intensities. As chlorophyll is a limiting factor for photosynthesis only at rather low concentrations (cf. Blaauw-Jansen et. al., 4), the photosynthetic yield can

increase with increasing light intensities in spite of pigment destruction. This explains why one gets a stomatal response at an intensity of 64,000 meter candles, although the pigment concentration is very low.

Discussion

Smith (17) was able to show that the initial chlorophyll a formed during a short period of illumination, which is derived from the transformation of the protochlorophyll originally present in dark-grown leaves, is photosynthetically inactive. As additional chlorophyll is formed in light, the oxygen-evolving capacity increases, at first about in proportion to the increase in chlorophyll. If the same conditions are valid for the chlorophyll formation and proto-synthesis in the guard cells of the stomata as in the mesophyll cells, this would imply that no photosynthetic material can be formed until more chlorophyll than that present after the first transformation from the proto-chlorophyll initially present has been formed. From the experimental data for the chlorophyll formation (Figure 2), it is obvious that the real increase in chlorophyll content comes first after 2—3 hours of continuous illumina-tion or just after the period which must elapse before the guard cells can show a response when illuminated continuously with an optimal light inten-sity. This interval also corresponds to the period of slow rate of photosyn-thesis of etiolated oat leaves at the beginning of illumination (Blaauw-Jansen et al., 4). From this the conclusion might be drawn that the conditions in the guard cells in respect to photosynthetic activity correspond to those valid for the chlorophyll-containing cells in general. It should be pointed out, however, that direct evidence for that photosynthesis takes place in the guard cells has never been presented (cf. Stålfelt, 21; pp. 413—414), although there are several indirect evidences for that photosynthesis really occurs in these cells, as will be discussed below. The fact that the chlorophyll resulting from the transformation of the protochlorophyll present in darkness lacks photo-synthetic activity seems to be due to some other light-dependent agent which must be simultaneously present for the photosynthesis to take place. The activity due to the presence of this agent increases considerably if the admi-nistered light is given in short periods instead of continuously (Smith, 17).

It was shown by Alvim (1) that stomata of green plants which have been in darkness for a long period of time do not respond immediately to a light impulse with an opening movement. If the plants have been in darkness several days it will take a few hours before any increase in transpiration can be shown, proving that an opening movement of the stomata has taken place. In the guard cells of plants so treated, the content of starch is low. In spite

of the fact that the guard cells in this case contain chlorophyll pigments, the light must act for a considerable period of time before any increase in the contents of photosynthetic material can be found. Whether this time lag also shown by Stålfelt (20) and Virgin (24) has anything to do with induction phenomena correlated to the aforementioned findings by Smith (17) is not known. From de Alvim's- (1) and other experiments (cf. Yemm and Willis, 26) the conclusion can be drawn, however, that an absolute pre-requisite for the occurrence of light-induced stomatal movements is the presence of photosynthetic material in the guard cells. There are no experimental evidences for that starch is deposited in darkness in the guard cells as a result of an accumulation of carbohydrates originating from other surrounding or underlying cells. One might therefore assume that the photosynthetic material present in the guard cells is derived from photosynthetic activity of the chloroplasts in the same cells. As discussed above the formation of this photosynthetic material is probably subjected to the same conditions as valid for the photosynthesis of that particular plant in general, although the equilibrium in darkness of starch $\rightleftarrows$ soluble carbohydrates in the guard cells of monocots is shifted more to the left than in the underlying mesophyll cells.

As long as the stomata are closed very small amounts of carbon dioxide can enter the leaf. This may be one of the reasons why photosynthetic material is formed so slowly in the beginning of illumination. Carbon dioxide derived from respiration can only play a rôle for photosynthesis if one presumes that it can diffuse from one cell and is used for photosynthesis in another — in this case the guard cell.

Many theories of the mechanism of the stomatal movements have been presented. They have recently been reviewed and critically discussed by Stålfelt (21; pp. 413—421). The data presented in the present paper support the view that the presence of chlorophyll pigments is of a certain importance for the occurrence of the opening movements. This should thus favour the hypothesis that photosynthesis in some way is involved in the mechanism. That such is the case is also strongly supported by the aforementioned behaviour of the stomatal starch in light and darkness. There is as yet no indication that the carbohydrates in the guard cells should not be the osmotic agent in the turgor movements. That pH-changes affecting the osmotic equilibrium also are involved in the mechanism has been shown by several authors (for references, see Stålfelt, 21; pp. 415—416). It is doubtful, however, whether these changes are derived solely from changes in the carbon dioxide content of the cells, as Liebig (9) has shown that the increase of pH in light in some cases can proceed so fast that it is not possible to correlate it directly with photosynthetic activity.

There are several data available concerning the effect of different light qualities on the opening movements. Although there are some discrepancies in the experimental results, most of the data point to blue light being more effective than it should be if only the chlorophyll pigments were responsible for the energy uptake (Harms, 6; Liebig, 9; Paetz, 11; Pyrkosch, 12; Sierp, 15). This would thus imply that a blue-sensitive process is involved in the mechanism and that there is a light-influence acting directly on the proto-plasm.

According to Bünning (5; pp. 425—430) part of the light-sensitive reaction is of an all-or-none type like the light-turgor reactions of the leaf pulvini, implying that it is partially independent of the administered light intensity. On the basis of experiments by Harms (6) he assumes that it is this part of the reaction which is connected to light energy absorbed by carotenoids.

It should be pointed out in this connection that an etiolated dark-grown plant lacks not only chlorophyll pigments. Also the formation of carotenoids is light-dependent. This formation seems to follow the same pattern as for chlorophyll a, *i.e.*, a very slow formation during the first 2 hours in light, whereafter an acceleration sets in (Blaauw-Jansen *et al.*, 4). The question whether one can obtain stomatal movements with only carotenoids present in plastids of the guard cells is therefore still unanswered. It may be that some of the questionable stomatal movements in variegated parts of green leaves (cf. the introduction) are due to light energy absorbed by yellow pigments.

The real rôle of other pigments than the chlorophylls in the stomatal movements can only be settled by an accurate determination of the action spectrum for the process.

Summary

The transpiration from etiolated wheat leaves has been measured and compared with the chlorophyll *a* content of the leaves.

Etiolated wheat leaves were illuminated with different intensities of light while the transpiration was measured. No stomatal transpiration could be detected before 2—3 hours after the beginning of the illumination, regardless of the intensity of the light. Strong intensities give proportionally lower transpiration values than medium intensities.

Very small amounts of chlorophyll *a* in addition to that formed from the protochlorophyll initially present are accumulated during the first 2—3 hours of illumination. Later on an acceleration in the formation sets in. Strong

intensities of light cause a partial destruction of the chlorophyll-forming mechanism.

The eventual rôle of photosynthesis in the stomatal mechanism is discussed.

References

1. Alvim, P. de T.: Studies on the mechanism of stomatal behaviour. — Amer. J. Bot. 36: 781. 1949.
2. Andersson, N. E. & Hertz, C. H.: Positive Spitzenentladung als Hygrometer geringer Trägheit. — Zeitschr. f. angew. Physik 7: 361. 1955.
3. — — & Rufelt, H.: A new fast recording hygrometer for plant transpiration measurements. — Physiol. Plant. 7: 753. 1954.
4. Blaauw-Jansen, G., Komen, J. G. & Thomas, J. B.: On the relation between the formation of assimilatory pigments and the rate of photosynthesis in etiolated oat seedlings. — Biochim. et Biophys. Acta 5: 179. 1950.
5. Bünning, E.: Entwicklungs- und Bewegungsphysiologie der Pflanze. — Lehrbuch der Pflanzenphysiologie, Bd. II, III. Aufl. III. Springer; Berlin 1953.
6. Harms, H.: Beziehungen zwischen Stomataweite, Lichtstärke und Lichtfarbe. — Planta 25: 155. 1936.
7. Koski, Violet M. K.: Chlorophyll formation in seedlings of Zea Mays L. — Arch. Biochem. 29: 339. 1950.
8. —, French, C. S. & Smith, James, H. C.: The action spectrum for the transformation of protochlorophyll to chlorophyll a in normal and albino corn seedlings. — Arch. Biochem. and Biophys. 31: 1. 1951.
9. Liebig, Margarete: Untersuchungen über die Abhängigkeit der Spaltweite der Stomata von Intensität und Qualität der Strahlung. — Planta 33: 206. 1942.
10. Lloyd, F. E.: The Physiology of Stomata. — Carn. Inst. Wash. Publ. 82. 1908.
11. Paetz, K. W.: Untersuchungen über die Zusammenhänge zwischen stomatärer Öffnungsweite und bekannten Intensitäten bestimmter Spektralbezirke. — Planta 10: 611. 1930.
12. Pyrkosch, G.: Licht und Transpirationswiderstand. — Protoplasma 26: 418. 1936.
13. Rufelt, H.: Influence of the root pressure on the transpiration of wheat plants. — Physiol. Plant. 9: 154. 1956.
14. Scarth, G. W.: Mechanism of the action of light and other factors on stomatal movement. — Plant. Physiol. 7: 481. 1932.
15. Sierp, H.: Untersuchungen über die Öffnungsbewegungen der Stomata in verschiedenen Spektralbereichen. — Flora 128: 269. 1933.
16. Smith, James H. C.: Protochlorophyll, precursor of chlorophyll. — Arch. Biochem. 19: 449. 1948.
17. — The development of chlorophyll and oxygen-evolving power in etiolated barley leaves when illuminated. — Plant Physiol. 29: 143. 1954.
18. — & Benitez, Allen: Chlorophylls: Analysis in Plant Materials. — In: Modern Methods of Plant Analysis. Vol. IV. Ed.: K. Paech and M. V. Tracey. Springer; Berlin 1955.
19. — & Young, Violet M. K.: Chlorophyll Formation and Accumulation in Plants. — In: Radiation Biology, Vol. III. Ed.: Alex. Hollaender. McGraw-Hill. New York 1956.

20. Stålfelt, M. G.: Die photischen Reaktionen im Spaltöffnungsmechanismus. — Flora N.F. 21: 235. 1927.

21. — Die Stomatäre Transpiration und die Physiologie der Spaltöffnungen. — In: Encyclopedia of Plant Physiology. Part II. Vol. III. Ed.: W. Ruhland. Springer; Berlin 1956.

22. Virgin, Hemming I.: The conversion of protochlorophyll to chlorophyll a in continuous and intermittent light. — Physiol. Plant. 8. 389. 1955.

23. — Protochlorophyll formation and greening in etiolated barley leaves. — Physiol. Plant. 8. 630. 1955.

24. — Light-induced stomatal movements in wheat leaves recorded as transpiration. Experiments with the corona-hygrometer. — Physiol. Plant. 9. 280. 1956.

25. Wilson, Ch. Chr.: The effect of some environmental factors on the movements of guard cells. — Plant Physiol. 23: 5. 1948.

26. Yemm, E. W. & Willis, A. J.: Stomatal movements and changes of carbohydrate in leaves of Chrysanthemum maximum. — New Phytologist 53: 373. 1954.

Effect of Indole-3-Acetic Acid on the Formation of Oxidases in Fungi

By

Gösta Fåhraeus and Viktor Tullander

Institute of Microbiology, Royal Agricultural College, Uppsala 7, Sweden
(Received July 21, 1956)

In earlier work from this Institute (2—5, 9, 10) the occurrence and forma-
tion of phenol oxidases in fungi has been studied. It was found that the
formation of laccase is readily induced by a number of substances, the struc-
ture of which is characterized by an OH— or NH_2— (NH—) group linked to
an aromatic nucleus. Most of these substances had a weak or moderate acti-
vity, while some, *e.g., o*-toluidine were strong inducers.

In subsequent work, further compounds of this general type have been
tested. Special interest was given to indole and its derivatives, since previous
experiments with the series tryptophan —- indole — anthranilic acid had
shown that indole was a very active inducing substance (5). In several expe-
riments it has now been established that indole-3-acetic acid (IAA) is more
effective than indole in this respect. Since there is recent evidence that IAA
influences the formation of some oxidases in higher plants (see discussion),
the similar effect of IAA also in fungi seems to deserve mention.

Materials and methods

Two fungi belonging to the Basidiomycetes, viz. *Polyporus versicolor* Fr. and
Marasmius scorodonius Fr. were studied in the experiments reported here. The strain
of *P. versicolor* was the same as used in earlier work. The strain of *M. scorodonius*
was the one studied by Lindeberg and Holm (10). Stock cultures of these species
were maintained on malt extract agar. In the experiments the following basal me-
dium was used:

Glucose 20 g, L-Asparagine 2.5 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7 H_2O$ 0.5 g, $CaCl_2$ 0.01 g,

$FeSO_4 \cdot 7 H_2O$ 0.01, $MnSO_4 \cdot 4 H_2O$ 0.001, $ZnSO_4 \cdot 7 H_2O$ 0.001, $CuSO_4 \cdot 5 H_2O$ 0.0001 g, DL-phenylalanine 0.15 g, aneurin. HCl 50 µg, dist. water 1000 ml.

The pH: about 5.5 after autoclaving.

This medium is only a slight modification of the media given in earlier publications (3, 4). It is referred to as medium Bf—2.

In most experiments 6 replicate flasks were set up each containing 20 ml of medium. The flasks were inoculated with a mycelium suspension obtained by shaking a 6 days culture with glass beads in a glass stoppered wide-necked bottle. The technique was similar to that recommended by Wikén et al. (14).

Usually after 6 days growth, the additions were made aseptically. The appropriate amounts of the substances to be tested were dissolved in 95 per cent ethyl alcohol, and 0.2 ml of the alcohol solution was added to each flask. The control flasks received 0.2 ml pure alcohol. In no case was the growth or enzyme production affected by the addition of alcohol alone.

After 10 days, the mycelia were filtered off, rinsed in water, dried at 100° C, and weighed. The standard errors of the mean values given in the Tables were calculated according to the formula

$$m = \sqrt{\frac{S\,(x - \bar{x})^2}{n\,(n-1)}}$$

In the experiment with the rather slow-growing *M. scorodonius* (Table 4) the flasks were harvested after about one month. The additions were in this case made at the beginning of the experiment (before inoculation). Mycelial extracts of this fungus were obtained as given earlier (3).

The oxidase determinations were made by means of the standard Warburg technique, catechol being used as the substrate. In special cases tyrosine, hydroquinone, ascorbic acid, or IAA were also tested as substrates. The vessels contained 1 ml 0.1 M acetate buffer pH 5.0, 1 ml culture dialyzate and 0.4 ml 0.1 M substrate solution. The determinations were run at 25° C. The oxidase activity was expressed as the amount of O_2 in µl taken up during one hour under these conditions. A difference in manometer readings of less than 2 mm/hr (corresponding to c. 50 µl/culture/hr) is deemed unreliable.

Experiments and Results

A preliminary experiment with *P. versicolor* and with indole, indole-3-acetic acid (IAA) and indole-3-butyric acid (JBA) in a 10^{-3} M concentration indicated that IAA was the most active of these three compounds. In two subsequent experiments IAA was then compared with various substances earlier found to induce laccase formation, the concentrations 5×10^{-4} and 1×10^{-4} M being tested. The results are given in Table 1.

As is evident from this Table, IAA in a 5×10^{-4} M concentration is one of the most powerful inducers. In the lower concentration (1×10^{-4} M) IAA turned out to be the most efficient addition, but too much importance should not be attached to these relative values, as the relation is not quite constant from one experiment to another. This is apparent from a later Table (Table 3).

Table 1. *Effect of various substances on growth and laccase production of Polyporus versicolor.* pH at the start 5.6, after 10 days 4.6.

Addition	Concentration of added substances					
	5×10^{-4} *M*			1×10^{-4} *M*		
	Mycelial dry weight in mg	Laccase activity μl O_2 per hour		Mycelial dry weight in mg	Laccase activity μl O_2 per hour	
		per culture	per mg mycelium		per culture	per mg mycelium
None, 6 days	67	—	—			
None, 10 »	164 ± 0.69	< 50	< 0.3	152 ± 0.69	50	0.3
Catechol	159 ± 1.6	580	3.6			
p-Cresol	161 ± 0.86	780	4.8	152 ± 0.98	50	0.3
Guaiacol	155 ± 2.0	3,200	21	156 ± 1.3	1,200	7.7
o-Aminophenol	160 ± 1.1	4,000	25	153 ± 1.1	102	0.7
o-Toluidine	171 ± 1.6	8,400	49	160 ± 1.8	920	5.8
o-Anisidine	174 ± 1.6	3,100	18			
α-Naphthol	165 ± 4.0	2,500	15			
β-Naphthol	173 ± 3.3	3,600	21	165 ± 2.2	460	2.8
8-Hydroxyquinoline				154 ± 0.73	1,100	7.1
Indole-3-acetic acid	166 ± 1.1	4,400	27	157 ± 1.6	1,500	9.5

It may suffice to say that substances such as IAA, 8-hydroxyquinoline, o-toluidine, and guaiacol are all very active.

None of the compounds given in the Table is so toxic as to suppress growth in the lower concentration used. This is important, since evidence has been presented that the laccase formation tends to increase at toxic concentrations of inducing substances (4).

As the concentrations of IAA used in these experiments far exceeds those

Table 2. *Effect of various amounts of IAA on growth and laccase production of P. versicolor.* pH at the start 5.5, after 10 days 4.8.

IAA, Mol/lit.	Mycelial dry weight in mg.	Laccase activity, μl O_2 per hour	
		per culture	per mg mycelium
None, 6 days	64 ± 1.5	—	
None, 10 »	148 ± 2.9	120	1
1×10^{-6}	152 ± 3.2	150	1
1×10^{-5}	155 ± 1.8	150	1
2×10^{-5}	161 ± 2.0	150	1
5×10^{-5}	160 ± 4.0	290	1.8
1×10^{-4}	157 ± 3.2	1,200	7.6
2×10^{-4}	157 ± 1.3	2,900	18
5×10^{-4}	155 ± 1.5	4,600 (1,200[1])	30
1×10^{-3}	156 ± 0.90	6,400	41
1×10^{-5} added at the start	130 ± 4.3	270	2.1

[1] With IAA instead of catechol as a substrate and in the presence of KOH in the Warburg measurements.

Table 3. *Effect of some auxins and related substances on growth and laccase production of P. versicolor.* pH at the start 5.8, after 10 days 4.6.

Series	Substance (10^{-4} M)	Mycelial dry weight in mg.	Laccase activity, µl O_2 per hour	
			per culture	per mg mycelium
1	None, 6 days	68 ± 3.4	—	—
2	None, 10 »	153 ± 3.0	0	0
3	Anthranilic acid	153 ± 3.6	0	0
4	o-Toluidine	159 ± 2.4	940	5.9
5	Indole	147 ± 3.9	0	0
6	Skatole	160 ± 2.4	34	0.2
7	Indole-3-acetic acid	162 ± 2.0	520	3.2
8	Indole-3-propionic acid ...	164 ± 3.0	270	1.6
9	Indole-3-butyric acid	151 ± 2.6	520	3.4
10	L-Tryptophan	155 ± 0.45	0	0
11	DL-Kynurenin (2×10^{-4} M)	158 ± 4.5	0	0
12	2-Naphthol	149 ± 2.6	170	1.1
13	2-Naphthoxy-acetic acid ...	152 ± 2.4	190	1.3
14	2-Naphthylamine	154 ± 3.4	730	4.7
15	2,4-D	155 ± 2.7	140	0.9

usually effective in growth experiments with higher plants, more dilute solutions of IAA were tried as well (Table 2).

From this Table it is clear that there is no appreciable stimulation of the laccase formation at concentrations below ca. 5×10^{-5} M IAA, if the addition is made after 6 days' growth, when the culture contains rather much mycelium. If, on the other hand, IAA is added before inoculation, a certain effect is observed even at a concentration of 1×10^{-5} M. In this case the growth rate is reduced to about 70 per cent of the normal value.

The enzyme formed on addition of 5×10^{-4} M IAA is also able to oxidize IAA, although the oxidation of catechol goes much faster.

In the next experiment the effect of IAA was compared with that of a number of other substances mainly belonging to the indole and naphthalene series of compounds. Some of these substances are known as plant auxins (nos. 7, 8, 9, 13, 15). Nos. 3—11 are structurally related to indole.

The results are presented in Table 3.

From the Table the fact is evident that IAA and IBA had the highest activity within the indole series of substances. However, the simple compound, o-toluidine, which is structurally related to indole, had in this experiment a still higher activity. The naphthalene derivatives were also active, particularly 2-naphthylamine. The two plant auxins, 2-naphthoxy-acetic acid and 2,4-dichlorophenoxy-acetic acid, had a definite although somewhat weaker effect.

In two series — with the addition of o-toluidine and IAA, respectively — manometric determinations were carried out with hydroquinone, tyrosine,

Table 4. *Effect of IAA and o-toluidine on growth and phenol oxidase production of Maras-mius scorodonius. Additions made at time 0. Harvested after 30 days.*
pH at the start 5.3, after 30 days 5.1.

Substance added	Mycelial dry weight in mg	Laccase activity, μl O_2 per hour		Tyrosinase activity μl O_2 per hour
		per culture	per mg mycelium	per culture
None	141 ± 1.1	990	7.0	< 1.5
IAA 10^{-3} M	3.5 ± 1.0	97	29	
IAA 10^{-4} M	148 ± 1.7	1,400	9.4	< 1.5
IAA 10^{-5} M	144 ± 1.8	1,600	11	
o-Toluidine 10^{-4} M	146 ± 1.8	1,700	12	

and ascorbic acid, as well as with catechol as substrates. This was done in order to rule out the possibility that the oxidase formed was tyrosinase or ascorbic acid oxidase. As a result of these tests it can be stated that tyrosine was not oxidized, while hydroquinone was oxidized somewhat faster than catechol and ascorbic acid much slower than catechol. This is rather typical for laccase (see also Dawson and Tarpley, 1). Thus there is no reason to assume the presence of other oxidases than laccase in the culture solutions studied.

For an additional experiment *Marasmius scorodonius* was chosen, since this fungus is known to produce both laccase (extracellularly) and tyrosinase (intracellularly) (Lindeberg and Holm, 10). It was cultured on the ordinary medium and the culture solution as well as the mycelium was analyzed for laccase and tyrosinase. The results of this experiment are given in Table 4.

From Table 4 it is evident that there is a considerable formation of laccase even in the absence of an exogenous inducer. Yet it is possible to increase the laccase formation by the addition of IAA or toluidine as in the case of *Polyporus versicolor*. A 10^{-3} M concentration of IAA inhibits growth almost completely, but apparently not the laccase production.

The tyrosinase production was in this experiment negligible. No additional effect of IAA could be observed.

Discussion

Indole-3-acetic acid and indole-3-butyric acid are among the most active of the substances inducing laccase formation in cultures of *Polyporus versicolor*. An appreciable effect of IAA is observed in the range 5×10^{-5} to 1×10^{-4} M, but the effect increases considerably at least up to about 10^{-3} M. The auxins, 2-naphthoxy-acetic acid and 2,4-dichlorophenoxy-acetic acid (2,4-D), have

also a definite, although weaker effect. On the other hand, a few more or less unrelated substances, *e.g.* o-toluidine, 8-hydroxyquinoline, and guaiacol, are very active as inducers of laccase formation.

A similar effect of IAA and o-toluidine was observed in an experiment with *Marasmius scorodonius*. The response was in this case less pronounced, as a considerable non-induced laccase formation took place. A slight production of tyrosinase by this fungus was established by Lindeberg and Holm (10). However, in the present investigation no tyrosinase was found even if IAA had been added to the culture medium.

The growth of *P. versicolor* and *M. scorodonius* was only slightly affected by moderate IAA concentrations (Tables 2—4). Growth stimulations of fungi by IAA have been reported to occur (for references, see Fraser 1953), but no definite conclusions as to the mechanism have been reached. No similarity between the well-known effects of IAA on higher plants and the growth response of fungi can be said to exist.

On the other hand, the effect of IAA on laccase formation in fungi constitutes an interesting parallel to the effect on oxidase formation in higher plants reported in some recent publications. Thus, Newcomb (1951) showed that an addition of IAA $(2 \times 10^{-5} M)$ increased the ascorbic acid oxidase activity of tobacco pith sections grown in vitro about 6-fold. Assays for tyrosinase and cytochrom oxidase showed that these enzymes were not formed as a response to the addition of IAA. Galston and Dalberg (1954) found an adaptive formation of "IAA oxidase" in etiolated pea seedlings. In this case both 2,4-D and 1-naphthalene acetic acid as well as IAA were effective inducers of the oxidase. Peroxidase is believed to be an essential component of this enzyme system, and Jensen (1955) measured the peroxidase activity in root sections of Vicia faba and found it to be strongly inducible by a treatment with IAA (conc: 10^{-7} to $10^{-8} M$). In this instance, however, the analytical data do not seem to rule out entirely the possibility of a phenol oxidase being the induced enzyme, since enzymes of the laccase type have almost exactly the substrate range characteristic for the system peroxidase $-H_2O_2$.

Laccase and ascorbic acid oxidase are both copper-containing enzymes and both oxidize ascorbic acid (Dawson and Tarpley, 1). There are consequently reasons to believe that these enzymes have certain structural features in common. This might also imply that their formation is affected by the same type of inducers.

In the publications cited there is no statement as to the effect of other substances than the auxins mentioned. In view of the results with fungi presented here, compounds like o-toluidine and 2-naphthylamine should perhaps deserve considerations as well. But even if such substances would be

ineffective in the case of higher plants, it is not impossible that the same mechanism of enzyme induction is involved. Differences regarding the activity of a particular substance may well be explicable through different composition of the cell wall and plasma membranes of fungi and higher plants, respectively, and the difference in permeability and adsorption properties which may be expected on these grounds.

It has earlier been found that the presence of OH— or NH_2— (NH—) groups is a necessary condition for a compound to induce laccase formation (4, 5). The effect of 2-naphthoxy-acetic acid and 2,4-D found in the present work is therefore remarkable considering the lack of such groups. Perhaps the acetic acid is easily split off and then an effect comparable with that of 2-naphthol and 2,4-dichlorophenol would be expected. This is true for the former substance. The latter compound has not yet been tried as an inducer, but several chlorinated phenols and amines are rather active (unpublished results).

As shown in Table 2, IAA is readily oxidized by the enzyme produced by *P. versicolor* on addition of IAA to the culture medium. Since laccase — like peroxidase — is little specific in respect to hydrogen donors, it is hardly necessary to suspect the presence of other oxidases than laccase, *e.g.*, a particular IAA oxidase in these cultures. Also horse radish peroxidase oxidizes IAA (12). It seems as if the IAA oxidase from *Omphalia flava* described by Sequeira and Steeves (13) has much in common with the laccase studied in the present work. According to Ray and Thimann (12), who studied the reaction catalyzed by the Omphalia enzyme, both an oxidation and a decarboxylation is involved. This is also true for the Polyporus enzyme, which is apparent from the fact that the manometric measurements of laccase activity give significant pressure changes only in the presence of a CO_2-absorbing liquid.

Summary

Several substances including indole-3-acetic acid actively induce laccase formation in cultures of *Polyporus versicolor* and *Marasmius scorodonius*. Reference is made to recent work showing that IAA induces the formation of ascorbic oxidase and peroxidase in some green plants.

References

1. Dawson, C. R. & Tarpley, W. B.: Copper oxidases. — In: J. B. Sumner & K. Myrbäck, The enzymes II, 1: 454. 1951.

2. Fåhraeus, G.: On the oxidation of phenolic compounds by wood-rotting fungi. — Ann. Roy. Agric. Coll. Sweden 16: 618. 1949.
3. — Formation of laccase by Polyporus versicolor in different culture media. — Physiol. Plant. 5: 284. 1952.
4. — Further studies in the formation of laccase by Polyporus versicolor. — Physiol. Plant. 7: 704. 1954.
5. — & Lindeberg, G.: Influence of tyrosine and some other substances on the laccase formation in Polyporus species. — Physiol. Plant. 6: 150. 1953.
6. Fraser, I. M.: The growth-promoting effect of indole-3-acetic acid on the common cultivated mushroom, Psalliota hortensis (Cooke) Lange forma albida Lange. — Austral. J. Biol. Sci. 6: 379. 1953.
7. Galston, A. W. & Dalberg, L. Y.: The adaptive formation and physiological significance of indoleacetic acid oxidase. — Amer. J. Bot. 41: 373. 1954.
8. Jensen, W. A.: The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. — Plant Physiol. 30: 426. 1955.
9. Lindeberg, G. & Fåhraeus, G.: Nature and formation of phenol oxidases in Polyporus zonatus and P. versicolor. — Physiol. Plant. 5: 277. 1952.
10. — & Holm, G.: Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some Hymenomycetes. — Physiol. Plant. 5: 100. 1952.
11. Newcomb, E. H.: Effect of auxin on ascorbic oxidase activity in tobacco pith cells. — Proc. Soc. Exp. Biol. Med. 76: 504. 1951.
12. Ray, P. M. & Thimann, K. V.: Steps in the oxidation of indole-acetic acid. — Science 122: 187. 1955.
13. Sequeira, L. & Steeves, T. A.: Auxin inactivation and its relation to leaf drop caused by the fungus Omphalia flavida. — Plant Physiol. 29: 11. 1954.
14. Wikén, T., Keller, H. G., Schelling, C. L. & Stöckli, A.: Über die Verwendung von Myzelsuspensionen als Impfmaterial in Wachstumsversuchen mit Pilzen. — Experientia 7: 237, 1951.

Root Growth Effects of Indan, Indene, and Thionaphthene Derivatives

By

HANS BURSTRÖM and BERIT A. M. HANSEN

Botanical Laboratory, Lund
(Received July 23, 1956)

Introduction

The structural requirements of auxins, initially formulated by Koepfli, Thimann, and Went (1938) have been amended several times, most recently by Jönsson (1955) on account of experiments with root elongation by Hansen (1954) and Burström (1955 a) (cf. also Hansen, Burström, and Teär 1955).

Starting from *racem*-α-phenyl-butyric acid which is an auxin in high concentration, a ring closure between the ω-carbon of the side chain and the 2-position of the ring should not jeopardize the activity according to Jönsson, unless the more or less planar structure of the compound were disturbed. We should in this way arrive at compound ring systems of 5- and 6-membered rings with a carboxyl in 3-position possessing auxin activity. The corresponding 2-carboxylic acids, on the contrary, should be inactive. In order to test this presumption experiments have been carried out on the root growth activity of some such ring systems with a carboxyl in 2- or 3-position. Few compounds of this type have been studied previously: they will be mentioned in the following discussion. The test method used has been the micro-test on root cell elongation in wheat seedlings employed in previous studies (cf. Burström 1955 a).

However, the investigation has led to the conclusion that it is hardly possible at present to define structural requirements more than has been done, owing to the nature of the root elongation, and the fact supported by the present study that the compounds of auxinic type do not act in only one

point in the root elongation mechanism. The investigation has, for that reason, led to an attempt to identify and classify the types of growth activities exerted by the compounds.

The following compounds have been studied:

indan-2-carboxylic acid (I)
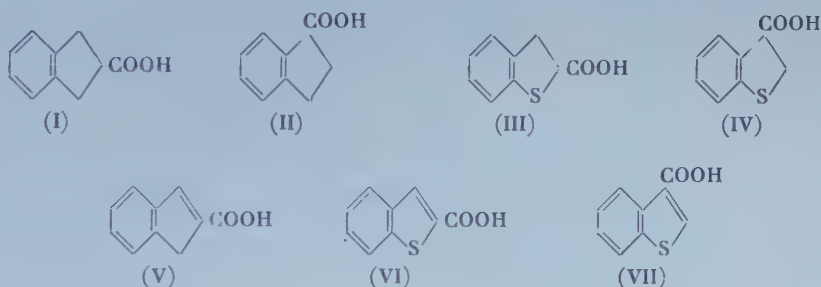*indan-3-carboxylic acid* (II)
dihydrothionaphthene-2-carboxylic acid (III)
dihydrothionaphthene-3-carboxylic acid (IV)
indene-2-carboxylic acid (V)
thionaphthene-2-carboxylic acid (VI)
thionaphthene-3-carboxylic acid (VII).

Compounds (I)—(IV), (VI), and (VII) were obtained from Professor A. Fredga, Chemistry Dept., Uppsala. compound (V) from Dr. Å. Jönsson, Dept. of Organic Chemistry, Royal Inst. of Technology, Stockholm.

Results

The results with the acids with a *carboxyl in 2-position* have been condensed in Figure 1. The details are not very clear, but, as a matter of fact, only two points of importance emerge from the experiments.

Firstly, that all acids are more or less active as cell elongation promotors in high concentrations, superficially resembling the erratic, and usually as inactive classified unsubstituted phenoxyacetic acid (Hansen 1954). Secondly, that particularly with indan-2- and indene-2-carboxylic acids the main root and the first pair of adventitious roots are remarkably unequally affected. The growth promotion is further unequally distributed within each root.

The wheat seedlings used have three well developed roots, which normally grow at the same rate with less than 5 per cent difference (Burström 1953); they are the main root and the simultaneously developing first pair of adventitious roots (roots nos. 2—3). Only the second pair of adventitious roots
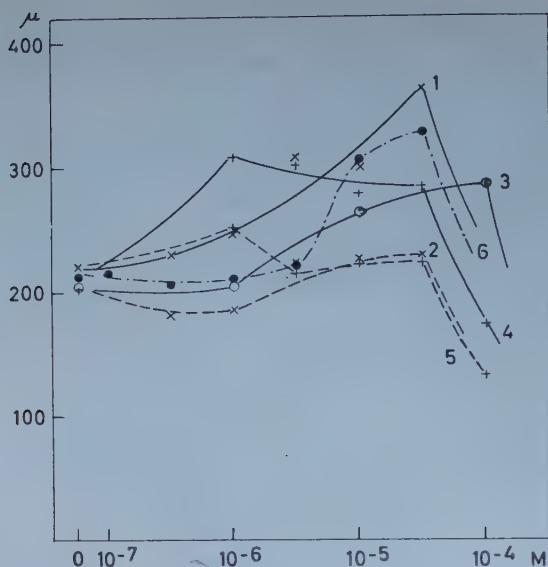
Figure 1. *The cell elongation activity of the -2-carboxylic acids.* Cell lengths in μ.

1 ×———— indan-2-CA root 1		*4* +———— indene-2-CA root 1
2 ×——— — root 2—3		*5* +——— — root 2—3
3 o ———— dihydrothionaphthene-2-CA		*6* ● ———— thionaphthene-2-CA

(nos. 4—5) might become less sensitive to growth regulators (Burström 1950). With the present compounds practically only the main root is affected and root nos. 2—3 grow normally.

The root epidermal cells are arranged in rows according to a more or less strict pattern: the cells are normally formed in pairs, the apical cell (the trichoblast) bears a root hair near its apical end, the basal cell of each pair lacks a root hair (the atrichoblast). Thus there follows in each pair of cells from the apical end basipetally: apical part of the trichoblast — point of insertion of the root hair — basal end of the same — atrichoblast. Trichoblast and atrichoblast are originally of practically equal length, and the root hair is initiated near the middle of that cell. The large final differences between the parts thus depend upon a normally occurring unequal elongation. This is called *differential elongation*, implying simply that the elongation does not proceed uniformly along the root axis. Zones, probably forming an irregular spiral around the root grow less than interjacent parts (Burström 1942). This can be demonstrated by using the cell boundaries and the points of insertion of the root hairs as fixed markings on the root surface, which can be done without preconceptions of the cause of the differentiation. The method has been employed for studying the differential elongation in the presence of some of the growth compounds of current interest.

Table 1 shows an example of the normal behaviour of the roots with regard to this differentiatial elongation and the effect of indan-2-carboxylic

Table 1. *The influence of indan-2-carboxylic acid $3 \cdot 10^{-5}$ M on the differential cell elongation.* — Lengths of cells and parts of cells in μ.

Material	Trichoblast			Atrichoblast	Average
	Apical part	Basal part	Total		
A. *Control*					
root 1	50 ± 3	133 ± 8	183 ± 7	268 ± 14	226 ± 8
roots 2—3	38 ± 1	145 ± 7	183 ± 7	243 ± 7	213 ± 5
B. *Indan-2-carboxylic acid*					
root 1	56 ± 4	182 ± 14	238 ± 13	356 ± 17	297 ± 11
roots 2—3	41 ± 3	153 ± 7	194 ± 7	282 ± 13	238 ± 8
Difference B—A					
root 1	*6 ± 5*	*49 ± 16*	*55 ± 14*	*88 ± 22*	–
roots 2—3	3 ± 3	8 ± 10	11 ± 10	*39 ± 15*	—

Differences significant at the 1 per cent level in italics.

acid. The acid increases the elongation of the atrichoblast of the main root, somewhat less the basal part of the trichoblast, and does not change the same parts of the roots 2—3. This strengthening of the differentiation is no new phenomenon, but has been encountered previously with growth enhancing concentrations of IAA and with α-indole-3-*iso*butyric acid. Pertinent data are collected in Table 2.

The table shows, firstly, the reproducibility of the behaviour of the plant material over a period of several years, and, secondly, the similar differential action of these three compounds. All growth compounds do not change the differential growth in this direction. On the contrary, inhibiting concentra-

Table 2. *Comparison between the differential promotion of the cell elongation by indole-3-acetic acid* (IAA), *α-indole-3-isobutyric acid* (IIBA), *and indan-2-carboxylic acid* (Indan-2-CA). Lengths of cell and parts of cells in μ. Mean errors ± 3 per cent.

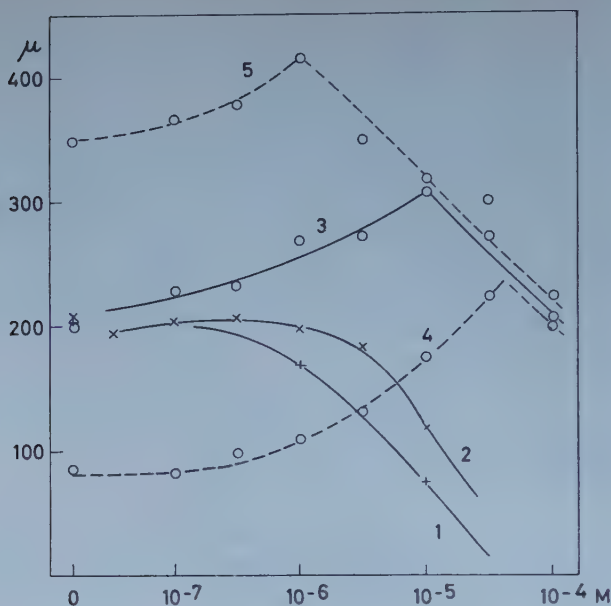| Material	Trichoblast			Atrichoblast
	Apical part	Basal part	Total	
A. Controls 1942	82	124	206	280
1954	60	125	185	258
1955	44	139	183	256
B. +IAA 10^{-10}—10^{-8} (1942)	80	143	223	350
IIBA 10^{-5} (1954)	58	183	241	421
Indan-2-CA 3.10^{-5} (1955)	49	167	216	319
Differences B—A				
IAA	— 2	19	17	70
IIBA	— 2	58	56	163
Indan-2-CA	5	28	33	64

Figure 2. *The cell elongation activity of the -3-carboxylic acids.* Cell lengths in μ.

1 +———— indan-3-CA
2 ×———— dihydrothionaphthene-3-CA
3 ○———— thionaphthene-3-CA
4 ○ — — —　　　　—　　　+1-naphthaleneacetic acid $3 \cdot 10^{-7}$ M
5 ○ — — —　　　　—　　　+α(4-chlorophenoxy)*iso*butyric acid 10^{-5} M

tions of IAA depress rather uniformly all parts of the root cells or preferr-ably the apical parts of the trichoblasts (Burström 1942, Burström and Han-sen 1956). This supports the assumption made in this paper that indole-3-acetic acid (an auxin) and α-indole-3-*iso*butyric acid (a root auxin) act on different points in the cell elongation mechanism. The similar activity of this acid and indan-2-carboxylic acid does not *prove* anything, but it leaves open the possibility that they are physiologically comparable and excludes the possibility that indan-2-carboxylic acid simply interferes with inhibiting IAA. More far-reaching conclusions are not justified.

As far as we are aware none of these compounds with a carboxyl in 2-position has been studied previously. Veldstra (1944) reports that the simi-lar indole-2-carboxylic acid and the 2-naphthoic acid (Veldstra 1949) are in-active in shoot tests. However, these tests were probably less sensitive to compounds of this physiological type.

The corresponding results with the *-3-carboxylic acids* are presented in Figure 2. The two acids with a saturated 5-ring, indan-3-carboxylic and
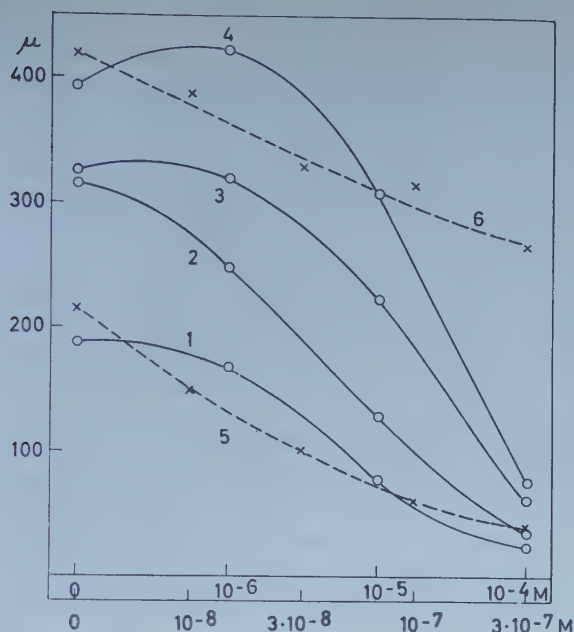
Figure 3. *The interaction between indan-3-carboxylic acid and α-indole-3-isobutyric acid.* As a comparison two graphs showing the interaction between indole-3-acetic acid and α-indole-3-*iso*butyric acid have been plotted in the diagram.

1	○ ———— indan-3-CA		*4*	○ ———	— + —	$3 \cdot 10^{-5}$
2	○ ————	— +α-IIBA 10^{-6} M	*5*	× — — — indole-3-acetic acid		
3	○ ———	— + — 10^{-5}	*6*	× — — —	—	+α-IIBA 10^{-5}

(Curves 5 and 6 from Burström and Hansen). On the upper abscissa the concentrations of indan-3-carboxylic acid, on the lower abscissa those of IAA. The graph has been constructed so that the control curves, nos. 1 and 5, coincide.

dihydrothionaphthene-3-carboxylic acids are similar growth inhibitors. In order to see whether this could be called an auxin action the indan-3-carboxylic acid was combined with α-indole-3-*iso*butyric acid (Figure 3). The curves show a rather ideal case of mutual interaction, starting with a slight synergism at the lowest concentrations, which often occurs. This is contrary to the mode of interaction between IAA and the *iso*butyric acid also plotted in the diagram. These act strictly addititively. If any conclusions can be drawn from combination experiments of this kind, it should be concluded that indan-3-carboxylic acid is a root growth inhibitor not physiologically identical with IAA, but acting in the same system as the *iso*butyric acid. It is doubtful whether it is justifiable to draw too rigid conclusions from results of this kind. The method has been employed in different connexions, also by the authors (Burström and Hansen 1956), but it is open to a certain criticism.

Table 3. *The influence of different inhibitors on the differential elongation.* Lengths of cells and parts of cells in μ. The concentrations chosen so as to give 25 per cent total reduction in elongation. Ind.-3-A=Indan-3-carboxylic acid.

Compound M	Trichoblast			Atrichoblast	Ratio atrichoblast: basal part
	Apical part	Basal part	Total		
Control	61	178	239	347	2.03
IAA $3 \cdot 10^{-8}$	29	144	173	265	1.87
1-NAA $6 \cdot 10^{-7}$	42	148	190	261	1.87
Ind.-3-A $3 \cdot 10^{-6}$ ..	52	146	198	263	1.92
Coumarin 10^{-5}	68	129	197	271	2.19
Diff. P=0.001	7	18	21	23	0.21

The evidences of the differential action are presented in Table 3. IAA, 1-NAA, usually regarded as physiologically identical with IAA, and indan-3-carboxylic acid resemble each other in that they show an exactly uniform inhibition of the atrichoblast and the adjacent basal part of the trichoblast. They differ, however, significantly in the inhibition of the apical part. Coumarin, interpreted as a non-auxinic inhibitor shows a very different behaviour.

Again conclusions must be very tentative, but the following ones seem to be reasonably well founded. *Indan-3-carboxylic acid and 1-NAA resemble generally IAA, but their actions are not exactly identical.* They may be supposed to act in two ways on the growth mechanism, one leading to a general inhibition, another to a differential elongation. The differences between the compounds may be only quantitative, IAA enhancing the differential elongation more strongly than the other two with the same general inhibition. — Fredga (1956) has reported indan-3-carboxylic acid to be active in routine tests, which, however, do not show the details of the mode of action.

Contrary to all expectations the thionaphthene-3-carboxylic acid is a root

Table 4. *The differential growth action of indan-2-carboxylic acid and thionaphthene-3-carboxylic acid.* Lengths of cells and parts of cells in μ. Mean error ± 2.5 per cent. (Statistical significance: between treatments and control P < 0.001, except for indan-2-carboxylic acid, apical part of trichoblast; between indan-2-carboxylic acid and thionaphthene-3-carboxylic acid P < 0.01, except between basal parts of trichoblasts.)

Material	Trichoblast			Atrichoblast
	Apical part	Basal part	Total	
1. Control 1956	42	157	199	283
2. Indan-2-carboxylic acid $3 \cdot 10^{-5}$	45	189	234	374
3. Thionaphthene-3-carboxylic acid 10^{-5}	61	192	253	342
4. 2+3	51	192	243	344

Figure 4. *The interaction between thionaphthene-3-carboxylic acid and indole-3-acetic acid.* — On the abscissa concentration of thionaphthene 3-carboxylic acid. Additions: *1* none, *2* IAA 3 · 10^{-8} *M*, cell lengths after 12 hours, *3 id.* cell lengths after 24 hours, *4* IAA 10^{-7} *M*, cell lengths after 12 hours, *5 id.* cell lengths after 24 hours.
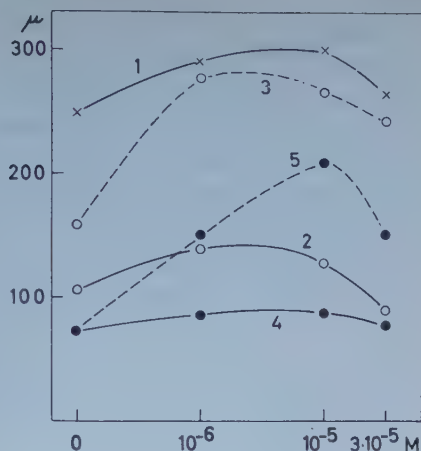
growth promotor (Figure 2). Combined with one auxin (1-NAA) and one root auxin it gave rather ideal additive interactions in both cases, which should be interpreted so that it is not comparable to either group of substances.

With regard to the differential action thionaphthene-3-carboxylic acid is of another type than that described in Table 2. This has been studied in several experiments with consistent results, exemplified in Table 4. The acid increases all parts of the cells uniformly, the action is practically non-differential and differs significantly from that of indan-2-carboxylic acid. This supports by implication the conclusion that it is not identical with the *iso*-butyric compounds either. Combined with indan-2-carboxylic acid it seems to dominate and is in a way superimposed on this acid.

It is always difficult to perform experiments of this kind with combinations of IAA, owing to the seeming adaptation to this acid. This is especially the case with thionaphthene-3-carboxylic acid as shown in Figure 4. IAA causes an immediate strong inhibition and a slow adaptation, in moderate concentrations complete after 48 hours. With the thionaphthene acid this adaptation is remarkably accelerated. Now it has been shown (Burström 1956) that this adaptation is not particularly related to the presence of IAA but depends upon a shift with time of the sensitivity to IAA, which has been interpreted as a normal change in the mode of elongation of roots passing from the juvenile to a more advanced state. The details are of little consequence in the present connexion. The crucial point is that *the normally occurring change in mode of growth is accelerated by thionaphthene-3-carboxylic acid.* The positive growth action of the acid itself may then be readily explained as a hastening of this change in character of the roots. This means

that the root growth promotion by this compound is not related to the common auxin or root auxin actions, a conclusion which is also borne out by the results recorded in Figure 2.

There are again only few relevant data in the literature. Koepfli, Thimann, and Went (1938) found indole-3-carboxylic acid inactive and Veldstra (1944, 1949) α-naphthoic acid only slightly active. Veldstra reports that α-(1,2,3,4,-tetrahydro)-naphthoic acid is more active on shoots and we have found a weak inhibition of roots resembling an auxin action, although it has not been analyzed further.

Discussion

According to Jönsson's (1955) requirements in compounds possessing auxin activity (i) the ring system should be planar, (ii) the carboxyl should be situated near the center of the ring, and (iii) the C of the carboxyl should be in the plane of the ring. According to a private communication by Dr. Jönsson, the studied compounds could be judged in the following way. Requirement (i) is fulfilled by indene-2- and thionaphthene-2- and -3-carboxylic acids. In those with a saturated 5-membered ring the deviation from the plane is probably unimportant so that this requirement should be more or less fulfilled by all compounds tested. According to (ii) the 3-substituted acids should be active, the 2-substituted less so. This fits in with the results insofar as none of the 2-substituted acids resembles an auxin, but all are root growth promotors.

Of the three 3-carboxylic acids the two without double bonds in the 5-membered ring should have a less favourable ring structure and an oblique orientation of the carboxyl; thus thionaphthene-3-carboxylic acid should be the most auxin-active compound. This does not hold true.

Within the rather heterogenous group of compounds more or less related to IAA there occurs all gradations of root growth activity, from strong growth promotion to strong growth inhibition. Usually in assessing activity growth inhibitions are supposed to imply auxin action — with due attention paid to unspecific toxic effects — and the more rare instances of root growth promotion are regarded as antiauxin actions. In between these extreme groups fall compounds denoted as weak auxins, weak antiauxins, or inactive. Especially the interpretation of the weakly active compounds has offered some difficulties (cf. Åberg 1952).

However, it has already been emphasized that a clear distinction between auxins and antiauxins is unwarranted, in that the antiauxins under certain circumstances and in some respects may behave as auxins (Burström 1955 b, Fawcett, Wain, and Wightman 1955, Hansen, Burström, and Tear 1955).

The present results have provided additional proofs of the fallacy of a stereotyped classification of growth-active compounds into auxins, non-auxins, or anti-auxins. It is obvious from a comparison between the growth inhibitors that their actions although superficially resembling that of IAA are not identical with it. This has been tentatively explained by referring to a growth mechanism involving two phases of growth with different actions of the compounds in the two growth phases. This theory has been elaborately developed in connexion with the action of growth regulators of non-auxinic nature, as Ca-ions and coumarin (Burström 1956).

The implications for a comparison between growth compounds may be exemplified by the modes of action of IAA, 1-NAA, and indan-3-carboxylic acid. *These acids exert histologically distinctly different actions,* although the inhibition of the root elongation may be the same. This could be interpreted so that the actions of the compounds are of different nature entirely, but such a far-reaching and, as far as IAA and 1-NAA are concerned, improbable conclusion is not necessary. Assuming that the compounds act in two ways on the growth mechanism the observations can be easily explained by quantitatively different actions of the compounds in the two systems. One is more active on one phase of the elongation, another on another part of the growth mechanism. *The observed differences between the compounds could in this way be reduced to only quantitative ones.*

The two manifestations of growth compared in this instance are *the net change in the cell length and the distribution of the elongation within the cell.* What this implies cytologically has not yet been elucidated nor its importance for the outcome of the elongation. It is certain, however, that these two parts are fairly independent of each other, although both affected by compounds of auxinic nature.

As to the relation between structure and activity the quantitative differences between the compounds may depend upon *partly different structural requirement for activity in the two systems.* This is, as a matter of fact, only a logical extension of the well-known fact that even good 'auxins' exert a variety of specific effects on undubitable growth phenomena such as root initiation, abscission, parthenocarpy, and so on. If it is only admitted that the normal cell elongation in itself involves several systems in which auxinic compounds interfere, it is not surprising that there is no generally valid relation between structure and activity of compounds, and that rather erratic exceptions from all rules occur.

The most spectacular one in the present material is that of thionaphthene-3-carboxylic acid. This ought to be a strong auxin, but is a root growth promotor, differing from the *iso*-butyric acid type. Its effect has been interpreted as *an acceleration of the normal course of development of the root from a*

juvenile stage to a more advanced condition. Roots of seedlings undergo
rapid developmental changes (cf. Pilet 1951) and in wheat plants these are
accompanied by a decreasing sensitivity to IAA, resembling an adaptation,
although it is not induced by the presence of IAA (Burström 1956). For this
or other reasons as well, the rate of elongation normally increases during
the earliest development of the seedlings, provided that conditions in general
are optimal. The action of thionaphthene-3-carboxylic acid may be explained
as an acceleration of this process, and a consequently increased root elonga-
tion over a short interval of time. Nothing similar has been detected as yet
with other compounds, and this seems to offer a good example of a part of
the growth mechanism with some specific structural requirements of its arti-
ficial regulator. The action must, of course, in some way be related to auxin
or the auxin conditions in the roots but not directly with the point of action
of auxin in the elongation mechanism. A further explanation of this effect
it at present not feasible, but it is an open question as to what extent other
instances of so-called 'weak anti-auxin' actions are of a similar nature.

The methods used for comparing the activities of compounds should be
commented on. Two ways have been chosen, neither of which is wholly
satisfactory. One is the well known method of combining two compounds
and studying the shape of the resulting concentration-activity curves, which
was introduced in this field by McRae and Bonner (1953) as a kinetical treat-
ment of the growth. In clear-cut instances it is not necessary to carry out
the double reciprocal computations for a rough evaluation of the data. It has
met with a certain criticism, and as regards root growth two points should
be added. The method rests on the assumption that growth can be regarded
as a chemical reaction proceeding — under favourable conditions — at a
constant rate. However, there is in the zone of elongation a number of cells
at any given moment growing at different rates, slowly near the apex, more
rapidly basally. Even if the root as a whole elongates at a constant rate this
is the integral of a number of different growth rates. This is elementary
knowledge of the course of root elongation. How coleoptiles behave in this
respect ought to be considered. Moreover, if the rate of root elongation is
changed, this often implies *a change in the duration of the elongation of the
individual parts not mainly of their rate of elongation* (cf. Burström 1956).
This seems to weaken the background of a kinetical treatment of growth
curves, and generally renders an evaluation of growth data rather delicate.

The other methods has been founded on the differential elongation and
the ensuing histological differentiation of the roots. If two compounds cause
different histological organization with the same total inhibition of the
elongation, it must be assumed that their actions are not identical in every
detail. This method of diagnosing activities is not unexceptionable either. It

can prove the existence but not the absence of differences in modes of actions, but if compounds have a superficially similar action, it fails. No method of assessing growth actions is infallible, and at present the only way seems to be to employ different methods with due attention paid to their weaknesses.

Summary

The influence on the root elongation of the following compounds has been studied: indan-2-carboxylic acid, indan-3-carboxylic acid, dihydrothionaphthene-2-carboxylic acid, dihydrothionaphthene-3-carboxylic acid, indene-2-carboxylic acid, thionaphthene-2-carboxylic acid, and thionaphthene-3-carboxylic acid. The compounds with a carboxyl in 2-position are well weak root growth promotors. Of the 3-substituted compounds indan-3- and dihydrothionaphthene-3-carboxylic acids resemble auxins, and thionaphthene-3-carboxylic acid is a root growth promotor. According to current theories all these three acids ought to be auxins.

Two methods for analyzing the mode of growth action have been employed and compared; by studying the effect of unknown compounds in combination with known ones, and by a histological study of the differential growth action. Neither method is wholly reliable.

The activity of the acids with a carboxyl in 2-position resemble root auxins of the iso-butyric acid type. The action of indan-3-carboxylic acid resembles that of IAA but they are not identical. Thionaphthene-3-carboxylic acid differs from both auxins and root auxins of the iso-butyric acid type; it is concluded that it promotes root growth by enhancing the normal development from the juvenile stage.

The results are discussed with regard to the mechanism of cell elongation and the problem of the structural requirements of growth regulators. It is emphasized that in diagnosing growth active compounds attention must be paid to the obvious heterogeneity of the mode of action on the cell elongation.

References

Åberg, B.: On the effects of weak auxins and antiauxins upon root growth. — Physiol. Plant. 5: 305. 1952.

Burström, H.: The influence of heteroauxin on cell growth and root development. — Ann. Agr. Coll. Sw. 10: 209. 1942.

— Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — Physiol. Plant. 3: 277. 1950.

— Studies on growth and metabolism of roots IX. Cell elongation and water absorption. — Physiol. Plant. 6: 262. 1953.

— Evaluation of the growth activity of naphthalene derivatives. — Physiol. Plant. 8: 174. 1955 a.

— Activity of plant growth regulators. — Ann. Appl. Biol. 42: 158. 1955 b.

— Auxin and the mechanism of root growth. — 1956. (in print).

— & Hansen, B. A. M.: The mode of growth action of some naphthoxy compounds. — In Wain and Wightman: The chemistry and mode of action of plant growth substances, p. 134. 1956.

Fawcett, C. H., Wain, R. L. & Wightman, F.: Studies on plant-growth regulating substances VIII. — Ann. Appl. Biol. 43: 342. 1955.

Fredga, A.: Zur Kenntnis der sterischen Spezifität pflanzlicher Wuchsstoffe. — Chem. Berichte 89: 322. 1956.

Hansen, B. A. M.: A physiological classification of 'shoot auxins' and 'root auxins'. — Bot. Not. 107: 230. 1954.

— Burström, H. & Teär, J.: Root and shoot elongation activity of some naphthalene compounds. — Physiol. Plant. 8: 987. 1955.

Jönsson, N. Å.: Synthetic plant hormones VIII. — Sv. Kem. Tidskr. 67: 166. 1955.

Koepfli, J. B., Thimann, K. V. & Went, F. W.: Phytohormones: structure and physiological activity. I. — J. Biol. Chem. 122: 763. 1938.

McRae, D. H. & Bonner, J.: Chemical structure and antiauxin activity. — Physiol. Plant. 6: 485. 1953.

Pilet. P.-E.: Contribution à l'étude des hormones de croissance (Auxines) dans la racine de Lens culinaris Medikus. — Mém. Soc. Vaudoise des sci. nat. 10: 137. 1951.

Veldstra, H.: Researches on plant growth substances. IV. Relations between chemical structure and physiological activity. I. — Enzymologia 11: 97. 1944.

— On the relation structure/activity with plant growth regulators. — Proc. 2nd Int. Congr. Crop Prot. 1949.

Photosynthesis in Heavily Centrifuged Algae [1]

By

K. A. Clendenning and T. E. Brown

Charles F. Kettering Foundation, Yellow Springs, Ohio
(Received May 15, 1956)

The centrifugations employed by Warburg and his associates (7, 8) in preparing Chlorella for quantum efficiency measurements have been very brief and mild in comparison to those customarily used in preparing algae and other microorganisms for metabolic studies: sedimentation was incomplete, and the thin layers of loosely packed cells resuspended when shaken gently for only a few seconds. Warburg, Burk and Schade (8) state that Chlorella is damaged by strong centrifuging, and that the cells may be partially destroyed during the resuspension of tightly packed sediments. »Such cell suspensions contain white Chlorella shadows and must be discarded.» (8) The present study was prompted by a need for quantitative information on after-effects of centrifugation treatments upon photosynthetic capacities which arose in a comparative study of Nostoc and Chlorella (4).

Chlorella pyrenoidosa, Emerson strain, was cultured in Warburg's medium (6) in a continuous culture apparatus similar to that of Benson *et al* (3). *Nostoc muscorum,* Gerloff strain, was similarly cultured in Eyster's medium (4). Cell numbers of both organisms doubled every 6—8 hours during the one-day cycles under 5 per cent CO_2 in air at 25° C. The centrifugation treatments employed in this study ranged from the mildest effecting quantitative sedimentation (10 min., 600 g.) up to three hours at 145,000 g., the maximum force obtainable with the Spinco preparatory ultracentrifuge. The »controls» were freshly harvested algae which had never been centrifuged. Since we were interested primarily in photosynthetic capacities, the cell suspension densities were lower than in efficiency

Table 1. *After-effects of centrifuging upon the photosynthetic capacities of C. pyrenoidosa and N. muscorum.* (ml. gas exchange per ml. packed cells per hour at 25° C.)

Pretreatment	Photosynthetic capacity	
	O₂ evolution	CO₂ assimilation
C. pyrenoidosa, 12 mm.³/vessel.		
1 (a) freshly harvested, uncentrifuged	92	84
(b) centrifuged 10 min. at 800 g., 25° C.	92	88
(c) centrifuged 20·min. at 25,600 g., 0° C.	92	89
C. pyrenoidosa, 7.25 mm.³/vessel.		
2 (a) freshly harvested, uncentrifuged	64	57
(b) centrifuged 30 min. at 1100 g., 25° C.	51	46
(c) centrifuged 20 min. at 24,500 g., 0° C.	67	60
N. muscorum, 7.25 mm.³/vessel.		
3 (a) freshly harvested, 10 min. at 800 g., 0° C.	29	28
(b) centrifuged 120 min. at 23,150 g., 0° C.	29	29
(same cells in 0.025 *M*. No. 9 buffer)		
4 (a) freshly harvested, 10 min. at 800 g., 0° C.	20	—
(b) centrifuged 120 min. at 23,150 g., 0° C.	20	—

measurements (ca. 10 mm.³ sedimentation volumes vs. 100 mm.³ upwards). Mechanical injuries after centrifuging were avoided by resuspending the sediments solely by shaking or by mild levigation with a glass syringe. Washing effects (4) were avoided by resuspending the cells in their supernate or in fresh nutrient. The photosynthesis measurements were completed within the second hour after centrifuging. The two-vessel measurements were made with a pair of rectangular (Machlett) vessels provided with gassing vents ($V_f = 5$ ml., $V_G = 10.3$, 17.17 ml.), the cells being suspended in their growth medium under 5 per cent CO_2 in air. Nostoc medium was supplemented with sodium bicarbonate (to 0.025 *M*.) prior to manometric equilibration under 5 per cent CO_2. The photosynthetic capacity measurements were commenced after ten minutes in saturating light, and were continued for 40—60 min. at 25° C.

Photosynthetic capacities were determined several times in freshly harvested, uncentrifuged, Nostoc and Chlorella, and in the same algae following mild centrifugations which effected quantitative sedimentation (10 min., 600—800 g., Clinical centrifuge, room temperature). The photosynthetic capacities of Nostoc and Chlorella were never reduced significantly by this standard treatment (Table 1). When Chlorella was centrifuged for 30 min. at 1100 g. in an International No. 2 centrifuge, the temperature of the cells rose from 25° to 32—33° C. After exposure to these conditions, the capacity for oxygen production and carbon dioxide assimilation was usually reduced about 20 per cent (Table 1); this was the largest after-effect of centrifugation which we have observed, and further work established that it was not a direct result of centrifugal force.

When Nostoc and Chlorella were centrifuged at 0° C. in a Servall angle

Table 2. *Photosynthetic capacities of C. pyrenoidosa before and after three hours of refrigerated centrifugation at 145,000 g.* (ml. gas exchange per ml. packed cells per hour at 25° C.).

Two-vessel measurements	Photosynthetic capacity	
	O_2 evolution	CO_2 assimilation
control (uncentrifuged)	42.6	35.7
centrifuged 3 hours at 145,000 g., 0° C.	40.1	34.7
Direct measurements in no. 9 buffer		
control (centrifuged 10 min. at 600 g.)	33.6	—
centrifuged 3 hours at 145,000 g., 0° C.	33.5	—

centrifuge, their photosynthetic capacities were not significantly reduced by lengthy exposure to *ca.* 25,000 g. (Table 1). The gelatinous sheaths on the Nostoc filaments caused adherence, making their sediments difficult to resuspend after heavy centrifugation. For this reason, effects of ultracentrifugation at 0° C. were determined only on Chlorella. Following three hours at 145,000 g., 0° C. the Chlorella sediment was free of white debris (8) and resuspended readily; the photosynthetic capacities either in complete nutrient under 5 per cent CO_2 or in 0.1 M. No. 9 buffer were not reduced significantly by this drastic centrifugation (Table 2). These findings exclude direct physical damage of Nostoc and Chlorella by centrifugal force (cell rupture, intracellular displacements) as a significant factor in photosynthetic capacity measurements following the shorter centrifugations at very much lower R. C. F. which are customarily used. Other evidence supports this conclusion: Chlorella cells are excepionally difficult to rupture for purposes of chloroplast isolation, (5); Elodea cells have been subjected to 350,000 g. for thirty minutes without more than 1 per cent cell breakage (1, 2).

The fact remains that photosynthetic efficiency (8) and capacity can be impaired by centrifugations which are too mild to cause direct physical damage. Such detrimental effects have only been observed with unrefrigerated centrifuges, operated at uncontrolled room temperatures. Overheating in the centrifuge can impair photosynthetic capacities, but this factor only becomes significant during lengthy centrifugations. When algae are centrifuged in the usual way at room temperature, their intracellular supply of oxygen is rapidly exhausted by respiration, the cells remaining in an anaerobic state until resuspended (the dark respiration rate of freshly harvested Chlorella and Nostoc at 25° C. is typically 1.0 and 0.5 ml. O_2/ml. p. c./hour respectively; dissolved oxygen within the cells in equilibrium with air amounts only to 4--5 mm.^{3}/ml. p. c., which must be replenished several times each minute to support respiration.). Photosynthesis is characteristically inhibited by preceding periods of dark anaerobiosis (anaerobic induc-

tion), if oxygen is not restored prior to illumination. The conditions within sedimentation cakes favor the accumulation of fermentation products, and here depth and compactness of the sediment as well as time and temperature should exert controlling influences. The fact that dark anaerobiosis in the sediment usually does not reduce the capacity for photosynthesis significantly is attributed to the rapid restoration of an aerobic state (oxidation of fermentation products) when the algae are resuspended. Possible complications from this source are eliminated by the use of mild centrifuging (8) and also are lessened by refrigerated centrifugation.

Summary

The photosynthetic capacity of the filamentous blue-green alga *Nostoc muscorum* was not reduced by refrigerated centrifugation for two hours at 23,000 g. The photosynthetic capacity of *Chlorella pyrenoidosa* was not reduced significantly by refrigerated centrifugation for three hours at 145,000 g. These observations are believed to exclude direct physical damage by centrifugal force as a significant factor in photosynthetic capacity measurements. Possible after-effects of overheating in the centrifuge and of anaerobic metabolism in tightly packed sediments are discussed.

References

1. Beams, H. W., Some effects of centrifuging upon protoplasmic streaming in Elodea. — Biol. Bull. 96: 246, 1949.
2. — & King, R. L.: The effect of centrifugation on plant cells. — Bot. Rev. 5: 132. 1939.
3. Benson, A. A., Calvin, M., Haas, V. A., Aronoff, S., Hall, A. G., Bassham, J. A. & Weigl, J. W.: Chapter 19. — Photosynthesis in Plants, edited by J. Franck and W. E. Loomis. Iowa State College Press, Ames, Iowa, 1949.
4. Clendenning, K. A., Brown, T. E. & Eyster, H. C.: Comparative studies of photosynthesis in Nostoc and Chlorella. — Canad. J. Bot. 34. 1956 (in press).
5. — & Gorham, P. R.: Photochemical activity of isolated chloroplasts in relation to their source and previous history. — Can. J. Research C 28: 114. 1950.
6. Warburg, O.: Messung des quantenbedarfs der photosynthese für sehr dünne zellsuspensionen. — Biochim. et Biophys. Acta 12: 356. 1953.
7. — & Burk, D.: The maximum efficiency of photosynthesis. — Arch. Biochem. 25: 410. 1950.
8. — — & Schade, A. L.: Symposia of the society for experimental biology, Number V, Carbon dioxide fixation and photosynthesis. p. 306. — Academic Press Inc., New York, 1951.

Causes of Increased and Stabilized Hill Reaction Rates in Polyethylene Glycol Solutions

By

K. A. Clendenning, T. E. Brown, and E. E. Walldov

Charles F. Kettering Foundation, Yellow Springs, Ohio
(Received May 15, 1956)

Introduction

Grana of blue green algae and chloroplasts of red algae effect water photolysis *in vitro* only when isolated in viscous solutions of polymers such as polyethylene glycols (10, 11) or dextrin (16). Phycocyanin and phycoerythrin (bilichromoproteins) (8) otherwise are dissolved, with accompanying inactivation of the water photolysis apparatus (10, 11). Thomas and De Rover (16) concluded that bilichromoproteins *in vivo* are located within the layered pigment-lipoprotein structures which effect water photolysis, and that their loss causes physical gaps and other damaging changes within the lamellae.

Bilichromoproteins have not been detected in higher plants, and it is usually unnecessary to employ polymeric solutes in isolating their chloroplasts in a photochemically active state. Nevertheless, McClendon (11) observed higher and more stable Hill reaction rates in chloroplasts from several higher plants when they were isolated and tested in 30 per cent Carbowax 4000 in place of 0.5 M sucrose. McClendon obtained this result consistently at the University of Minnesota, but in corresponding experiments at the Hopkins Marine Station, chloroplasts from several land plants as well as from Bryopsis failed to show beneficial effects of Carbowax upon the Hill reaction (11). Bishop, Lumry and Spikes (1) observed no preservative action of polyglycols and other polymeric solutes upon leaf chloroplasts stored at low temperatures.

[519]

The purpose of the present study was to learn why polyethylene glycols such as Carbowax 4000 had caused increased rates of water photolysis in some of the earlier experiments with leaf chloroplasts, and why this stimulatory or stabilizing action had not been observed consistently.

Methods

The main changes in the conditions of chloroplast isolation and testing with reference to those employed by McClendon (11) were the use of a uniformly low temperature in isolating the chloroplasts, and the use of a higher light intensity in measuring their photochemical activity; dilute chloroplast suspensions also were employed to ensure that chloroplast-limited conditions were provided.

Freshly harvested plant parts were brought to a cold room (0° C.). Weighed samples of cleaned leaf blades were immersed in ice-cold maceration media prior to chloroplast isolation at 0° C. The media were prepared by dissolving Carbowax,[1] sucrose, etc., in 0.05 M phosphate, pH 7.0, containing 0.01 M KCl. The leaves were ground thoroughly with sand (5 gm. leaves, 20 ml. grinding fluid (11). The brei was squeezed through a heavy pad of cheesecloth: the filtrate was centrifuged briefly at low speed, and the bulk of the chloroplast substance was then sedimented at 20,000 g. (10 min.). The supernate was retained for tannin analyses. The chloroplast sediment was dispersed in 20 ml. fresh medium (11). After providing uniform chlorophyll (9) concentrations by dilution, the suspensions were used in Hill reaction measurements. Further washing did not alter the effect of Carbowax vs. sucrose. The effects of Carbowax discussed below are shown with whole and fragmented chloroplasts, so these were not separated. Redispersion of chloroplasts in Carbowax solutions at 0° C. was always laborious, and when clumps could not otherwise be eliminated, they were removed by cloth filtration prior to chlorophyll analysis. The isolated chloroplasts were maintained at 0° C. for ca. two hours until the manometric rate measurements were commenced (usually at 10° C.). The Warburg vessels contained 2.5 ml. chloroplast suspension, with 0.5 ml. water containing 1 mg. resublimed p-benzoquinone in a masked sidearm. After equilibration and tipping, the vessels were illuminated with uniformly intense orange-red light (520—680 mμ, 70.000/ergs/cm.²/sec. at vessel). The light sources were closely spaced 300-w. projector floodlamps placed four inches below the horizontal window of a refrigerated shelftype thermostat. The light was filtered throught two ¹/₄ inch layers of heat-absorbing glass which were cooled by an air blast. Wavelengths shorter than 520 mμ were removed in the bath with a plastic filter. This equipment provided accurate temperature control (±0.02° C.) down to 5° C., and accommodated up to five reaction vessels. Water photolysis per hour per mgm. chlorophyll ($Q_{O_2}^{ch.}$) was calculated from the oxygen evolved during the first twenty minutes in light. Heat-inactivated controls showed no »activity» under these conditions. Provision of oxygen-free and CO_2-free atmospheres (vs. air) did not affect the rate measurements and so was

[1] Trade name for a polyethylene glycol supplied by Carbide and Carbon Chemicals Company. When 40 gm. is dissolved in 100 ml. phosphate buffer (11), the resulting Carbowax concentration is 30 per cent w/v due to liquid expansion.

usually omitted. Although quinone served as our standard oxidant, the main findings were confirmed with ferricyanide.

Dissolved tannins were determined in the supernatant extracts as follows. The extract was freed of proteins by heat coagulation (one min. at 100° C.) and centrifugation. The deproteinized supernate was acidified to pH 4.0 with 1 N HCl and analyzed by a modification of the A. O. A. C. method for tannin in wine (13). Collagen (American Standard Hide Powder) was substituted for bone black because of its greater specificity as a tannin-adsorbent. The collagen was prepared for use with chrome alum (13) and was stored moist (70 per cent water) in a sealed container at 0° C. The collagen/leaf extract ratio was varied to effect >95 per cent tannin removal, 0.3 gm. collagen solids usually being adequate for 5 ml. aliquots of leaf extract. The suspension was shaken mechanically for 10 min., centrifuged, the collagen was washed three times with water, and the combined eluate was made to known volume. An aliquot of leaf extract from which the tannins had not been removed was made to the same volume. Loss in $KMnO_4$ titre attending tannin removal was used in calculating tannin content (1 ml. $^1/_{10}$ N $KMnO_4 = 4.16$ mg. tannin (13). This procedure could not be applied to Carbowax 4000 extracts because this polyethylene glycol blocks tannin adsorption by collagen. The leaf extracts also were tested qualitatively for tannins with ferrous sulfate and ferric chloride solutions, and in some cases by precipitation tests with 1 per cent gelatin in 10 per cent NaCl. Vacuolar acidity was assessed by grinding leaves thoroughly with sand in two parts of distilled water and measuring the pH. Paraffin-embedded microtome sections of intact leaves were examined microscopically for tannin deposits after staining with 3 per cent ferrous sulfate (T. E. Brown, unpublished).

Results

Affinity of Carbowax for Tannin

Graded amounts of collagen were dispersed in 30 per cent Carbowax 4000 and 0.5 M sucrose solutions containing equal amounts of *Rhus typhina* leaf tannin. The suspensions were shaken mechanically for 10 min., centrifuged, and the supernates were tested for unadsorbed tannin. The Carbowax solutions gave positive tests for tannin following treatment with three times more collagen than was required for the complete removal of tannin from sucrose solutions. The following experiment showed that Carbowax also inhibits tannin adsorption by chloroplasts.

Ailanthus chloroplasts were isolated, washed and resuspended in 0.5 M sucrose. Aliquots containing 1 mg. chlorophyll were centrifuged at high speed and the sediments were dispersed uniformly (a) in 5 ml. 30 per cent Carbowax 4000 and (b) in 5 ml. 0.5 M sucrose, both at pH 4.0. Rhus extract (5 mg. tannin/ml.) was added in 0.25 ml. increments. After each addition, the mixtures were shaken mechanically 15 min., the chloroplasts were sedimented completely by centrifugation, and 0.25 ml. supernate was tested externally with 1 ml. 3 per cent $FeSO_4$. After resuspending the chloroplasts, a further 0.25 ml. of Rhus extract was added. The above procedure

was repeated four times. The color tests for unadsorbed tannin were consistently stronger in the presence of 30 per cent Carbowax 4000. The same result was obtained when the procedure was reversed by adding chloroplasts stepwise to a fixed amount of Rhus leaf tannin in 30 per cent Carbowax 4000 vs. 0.5 M sucrose.

The removal of leaf tannins from aqueous solutions by liquid-liquid extraction with ethyl acetate also is blocked by 30 per cent Carbowax 4000. Polyethylene glycols therefore must have considerable affinity for tannins in aqueous solution. This property of Carbowax 4000 was probably responsible for all of the foregoing phenomena as well as for its frequently observed inhibition of enzymatic browning in leaf macerates (which results from the action of polyphenol oxidase upon tannins and related compounds (14, 17).

Tannins are irreversible inhibitors of the Hill reaction which are commonly present in leaves (5). Since tannin adsorption by chloroplasts is impeded by Carbowax 4000, one might expect to obtain photochemically active chloroplasts from certain species with this reagent, though the same leaves yield essentially inactive chloroplasts when isolated in sucrose solutions, etc. Several examples of this favorable effect of 30 per cent Carbowax 4000 were encountered (*Quercus rubra, Prunus serotina, Acer saccharum, Robina Pseudo-Acacia* and *Geranium* leaves). The use of Carbowax, however, does not ensure that chloroplasts freshly isolated from tannin-bearing leaves will always possess photochemical activity *in vitro*. Thus *Ambrosia artemisifolia, Rhus typhina* and *Trifolium pratense* leaves yielded inactive chloroplasts consistently when 30 per cent Carbowax 4000 was employed. The chloroplast-inactivating agent in these leaves was shown to be soluble tannins with the aid of collagen. When leaves contain a high concentration of tannin already dissolved in the vacuoles, their chloroplasts are apparently inactivated before being released to the maceration medium. The largest improvements in the chloroplasts' initial capacity for water photolysis attending the use of Carbowax 4000 were observed, however, when the chloroplasts were isolated from leaves containing tannin.

Chloroplast inactivation by acidic cell sap (pH<4.0) is unaffected by Carbowax or by phosphate buffer in the grinding medium. Thus the »acid» leaves of *Ginkgo biloba* and *Oxalis stricta* yielded inactive chloroplasts when ground in buffered Carbowax as well as sucrose solutions, though the supernates gave negative tests for tannin. When Oxalis leaves were ground in distilled water, the pH of the extracts was always below 3.0, and »active» chloroplasts lost their capacity for the Hill reaction when briefly immersed in such extracts. Upon adjusting the Oxalis extracts to pH 7.0, their inhibiting action disappeared completely, showing that it is caused entirely by acidity (H$^+$). The chloroplasts of acid plants such as Oxalis are inactivated during isolation by exposure to the vacuolar acids before the cell contents are released to the buffered grinding fluid (5).

Chloroplast Stabilization by Carbowax

When tannin-free leaves having almost neutral cell sap (*e.g.* spinach) are ground in water or sugar solutions, their cytoplasm is dissolved so that it is easily separated from the chloroplasts. When leaves contain an abundance of tannins (*e.g.* Rhus) or when their cell sap is strongly acidic (*e.g.* Oxalis) cytoplasm is precipitated on the chloroplasts, which are simultaneously inactivated by these denaturants. Reference was made previously to this phenomenon by Waygood and Clendenning (18). Regardless of leaf tannins and acids, cytoplasm is precipitated on the chloroplasts when leaves are ground in 30 per cent Carbowax 4000. (The supernate is devoid of heat-coagulable protein.) This »salting-out» of the cytoplasm is reversed when the chloroplast-cytoplasm sediment is dispersed in neutral 0.5 M sucrose or 0.05 M phosphate. 30 per cent Carbowax 4000 prevents bilichromoproteins from dissolving (11), but it has a related effect on leaf cytoplasm, as well as on fresh egg albumen, which flocculates and settles in this solution. Low content of ultra-violet absorbing material in 30 per cent Carbowax 4000 supernates observed by McClendon (11) is a result of cytoplasmic insolubility in this reagent.

When whole or broken plastids are extracted with 30 per cent Carbowax 4000, they remain embedded in cytoplasm, so they are not »isolated» to the same degree as when 0.5 M sucrose is employed. Carbowax stabilizes the chloroplasts' capacity for water photolysis by coating them with cytoplasmic protein, an effect which is superimposed on the Carbowax-tannin interaction already discussed.

In our studies of chloroplast stabilization by Carbowax-cytoplasm, we investigated (a) »initial» capacities for water photolysis in chloroplasts from leaves having widely different solids and tannin contents, (b) storage deterioration and (c) Hill reaction rates over a wide range of temperatures.

(a) *Initial capacities for water photolysis*

To observe the chloroplasts' full capacity for water photolysis *in vitro,* it is necessary to use as dilute suspensions as when measuring the photosynthetic capacities of algae. Effects of mutual shading have been nullified in Hill reaction studies with *Chlorella* (2), but this has seldom been true in corresponding studies of isolated chloroplasts. (Concentrations of > 0.5 mgm. chlorophyll per Warburg vessel have commonly been used, which is 5—10 times higher than is employed in measuring photosynthetic capacities of algae.) When successively smaller chloroplast aliquots are subjected to intense light, the initial Hill reaction rate per unit of chlorophyll increases until mutual shading is eliminated (3). Simultaneously, thermal deteriora-

Table 1. *Effect of chloroplast density upon the initial photochemical activity of chloroplasts prepared in 30 per cent Carbowax 4000 and in 0.5 M sucrose.*

Chlorophyll, mgm/vessel	Carbowax	Sucrose
Ailanthus altissima	mm^{3}O$_2$ per hour per mgm. chlorophyll	
0.26	310	295
0.05	735	505
Medicago sativa		
0.50	525	530
0.15	815	625

tion becomes increasingly important. It is under these chloroplast-limited conditions that the stabilizing action of Carbowax-cytoplasm upon "initial" capacities for water photolysis is most evident.

Several examples were encountered (spinach, alfalfa, Ailanthus) of chloroplast suspensions which showed no higher initial activities when isolated in 30 per cent Carbowax 4000 vs. 0.5 *M* sucrose. Upon using smaller aliquots from the same chloroplast suspensions, the initial capacities for water photolysis at 10° C. (0—20 min.) regularly became higher in the Carbowax preparations (Table 1). McClendon (11) observed higher initial rates in chloroplasts isolated in Carbowax vs. sucrose at the University of Minnesota, but not at the Hopkins Marine Station; we obtained this result by using different amounts of chloroplasts from the same suspension (Table 1).

Table 2 reports rates of water photolysis for chloroplasts isolated in three media from twelve species. The chloroplast sources included leaves of trees and of small aquatic plants, whose water contents ranged from 46 per cent in Gleditsia to > 90 per cent in Lemna and Elodea. Although we did not measure the osmotic pressures within these chloroplast sources, it is known from earlier work that those within tree leaves are much higher than in herbaceous land and aquatic plants (6, 12). Since the stabilizing action of Carbowax 4000 was apparent in chloroplasts obtained from all of these sources, this effect cannot be dependent on the osmotic environment of the chloroplasts *in vivo*, which is far from constant even in a single leaf (6, 12). Smaller amounts of chloroplasts, longer storage and reaction periods, and higher isolation and reaction temperatures all tend to exaggerate the activity differences between Carbowax and sucrose preparations: the differences shown in Table 2 for Carbowax vs. sucrose could be made larger or smaller by manipulating these factors. In agreement with earlier studies, the initial Hill reaction capacities of chloroplasts isolated in neutral 0.5 *M* sucrose vs. 0.05 *M* phosphate were similar.

The acidities of the maple and red bud leaf saps might have caused slight inactivation of the chloroplasts during their isolation, but the acidities other-

Table 2. *Hill reaction activity of chloroplasts freshly isolated and resuspended at $0°$ C. in 0.05 M phosphate-buffered 30 % Carbowax 4000, 0.5 M sucrose, and control at pH 7.0. Tested with 1 mgm. quinone at $10°$ C. in orange-red light (70,000 ergs/cm^2/sec. at vessel). Activity calculated from O_2 evolution during first twenty minutes in light.*

Chloroplast source	% solids	Cell sap pH	Tannin mgm./ml. supernate	Chlorophyll mgm./ves-sel	$mm^3 O_2$/hour/mgm. chloro-phyll at $10°$ C.		
					Carbo-wax 4000	Sucrose	Phos-phate
Acer saccharum (Sugar maple)	43	4.2	4.6	0.2	350	105 [1]	65 [1]
Robinia Pseudo-Acacia (Black locust)	47	6.6	2.7	0.2	250	60 [1]	45 [1]
Cercis canadensis (Red Bud)	36	4.6	0.7	0.1	615	310	175
Gleditsia tricanthos (Honey locust)	54	5.3	0.4	0.2	985	535	510
Gymnocladus dioica (Kentucky coffee tree)	37	5.8	0.3	0.3	710	385	385
Ailanthus altissima (Tree of Heaven)	27	5.7	0.3	0.2	395	260	310
Medicago sativa (Alfalfa)	31	6.3	0.3	0.2	815	625	550
Trifolium repens (white clover)	25	6.2	0.3	0.2	545	395	400
Elodea canadensis (water weed)	9	6.8	0.2	0.2	600	450	310
Lemna minor (Duckweed)	6	6.9	0.2	0.1	540	395	350
Phytolacca americana (Pokeweed)	13	6.2	0.2	0.2	960	665	785
Spinacea oleracea (market spinach)		6.8	0	0.1	730	350	290

[1] Inactive after 15 minutes.

wise were too low to have significant effects. The reported tannin concentrations refer to the chloroplast suspensions which were prepared in phosphate buffer at $0°$ C. and used in Hill reaction measurements. The quantities of tannin extracted during chloroplast isolation vary with the isolation procedure (e.g. more tannin is extracted when leaves are ground at room temperature than at $0°$ C.). The tannin concentrations were highest in the maple and black locust preparations (from mature trees), which exhibited the lowest Hill reaction capacities, as well as the largest improvements through the use of Carbowax. After fifteen minutes in light, the chloroplasts from both these species were completely inactive when isolated and tested in 0.5 M sucrose or 0.05 M phosphate; water photolysis was far more stable in the Carbowax preparations. These are examples of Carbowax-cytoplasm stabilization superimposed on the Carbowax-tannin interaction. Leaves of small black locust seedlings differed strikingly from those of the mature trees in being

essentially free of tannins and in yielding chloroplasts with as high Hill reaction capacities as those of honey locust leaves (Gleditsia).

Every species found to contain even traces of tannin by the collagen method possessed microscopically visible tannin deposits within the leaves (stained microtome sections). Among the twelve species listed in Table 2, spinach alone was completely free of tannins, although the trace amounts found in the majority of the supernates would have only small effects on chloroplast activity. Apart from the maple and black locust data, the improved Hill reaction capacities observed in the Carbowax preparations (Table 2) must have been chiefly a result of Carbowax-cytoplasm stabilization, since the improvement observed in spinach chloroplasts was as large as in those from most of the other species.

Elodea is one of the several plants which have yielded »active» chloroplasts in one survey (11) and not another (4), which is most likely caused by varying concentrations of natural inhibitors. The Elodea samples used in the present study yielded active chloroplasts consistently, with evidence of the usual preservative effect of Carbowax-cytoplasm (Table 2). Takada (15) has provided information on the tannin-containing »idioblasts» of *Elodea densa*. Microscopic examination of the *Elodea canadensis* leaves employed in this study revealed the presence of similar tannin-containing cells, which were restricted to the margins of the leaf tips (T. E. Brown, unpublished studies).

Table 2 lists a number of new sources of »active» chloroplasts, which are mainly leaves of woody and herbaceous legumes. Several of these have proved suitable for research applications (Ailanthus, Gleditsia, Gymnocladus, Medicago).

(b) *Storage deterioration*

Losses in the Hill reaction capacity of alfalfa (Medicago) chloroplasts during their storage at $0°$ C. in Carbowax vs. sucrose have been compared at different chloroplast densities, in the presence and absence of cytoplasm. The experiment reported in Fig. 1 was based on chloroplasts isolated according to McClendon: 15 gm. freshly harvested alfalfa leaves were ground in 60 ml. neutral 30 per cent Carbowax 4000; uniform aliquots of the resulting chloroplast suspensions were centrifuged, and the supernates were removed completely. The »chloroplast-cytoplasm» sediments were dispersed in the original volume of (a) fresh 30 per cent Carbowax 4000 and (b) 0.5 M sucrose. The chlorophyll concentration was adjusted to 0.25 mg. chlorophyll/ml. These suspensions contained uniform amounts of leaf cytoplasm, (a) precipitated on the chloroplasts in 30 per cent Carbowax 4000, and (b) dis-
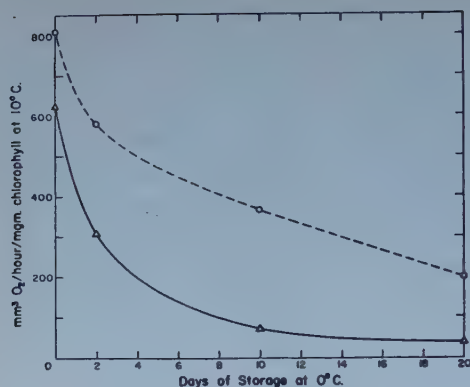
Figure 1. *Storage deterioration of water photolysis capacity in alfalfa chloroplasts isolated in 30 per cent Carbowax 4000 and resuspended in* (a) *30 per cent Carbowax and* (b) *0.5 M sucrose. Stored 9 days at 0° C. (0.5 mg. chlorophyll per conical Warburg vessel).*

　　o - - - - - o 30 per cent Carbowax 4000
　　△————△ 0.5 M. sucrose
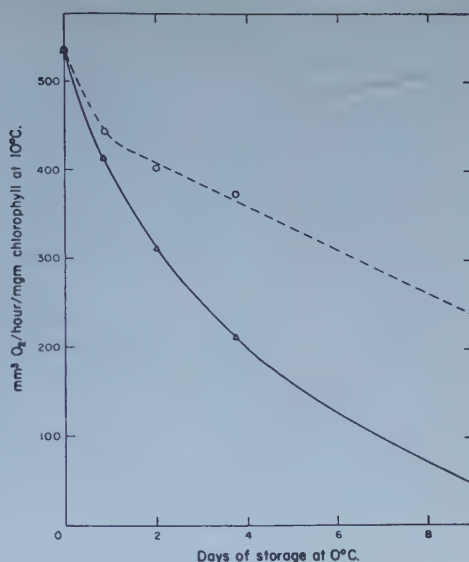
solved in 0.5 M sucrose. A relatively high chloroplast density was selected for this experiment (0.5 mgm. chlorophyll/vessel), the capacities for water photolysis being initially the same in Carbowax vs. sucrose. During nine day's storage at 0° C., the stabilizing action of the Carbowax became increasingly apparent (Figure 1). The storage half-life was about three times longer in Carbowax (ca. 7 days). Although the measured activities were initially identical, the $Q_{O_2}^{ch}$ values after nine days at 0° C. were 240 in Carbowax vs. 50 in sucrose. Corresponding experiments with other media showed similar deterioration rates in 0.5 M glucose and sucrose, and appreciably higher deterioration rates in 0.35 M NaCl, 0.05 M phosphate and 0.1 M sorbitol borate.

Figure 2 reports a related experiment. The alfalfa leaves were again ground in 30 per cent Carbowax 4000, but the chloroplasts which were stored in 0.5 M sucrose were first freed of cytoplasm by thorough washing in the latter solution. Smaller chloroplast aliquots were employed, the preservative effect of Carbowax-cytoplasm being apparent at the outset of the experiment (0.15 mg. chlorophyll/vessel). The storage period was lengthened to twenty days. The photochemical activities were higher in this experiment (Figure 2 vs. Figure 1) because of the lower chloroplast density, with consequent reduction of mutual shading. The storage half-life was again more than three times longer in Carbowax (ca. 7 days). After 20 days' storage at 0° C., the Hill reaction capacity in Carbowax-cytoplasm was still remarkably high $(Q_{O_2}^{ch}=200)$.

Figure 2. *Storage deterioration of water photolysis capacity in alfalfa chloroplasts isolated in 30 per cent Carbowax 4000, then washed and resuspended in* (a) *30 per cent Carbowax* (b) *0.5 M sucrose. Stored 20 days at 0° C., tested at 10° C. (0.15 mgm. chlorophyll per conical Warburg vessel.)*

o - - - - o 30 per cent Carbowax 4000

△————△ 0.5 M. sucrose

A third storage experiment was conducted on alfalfa chloroplasts which were isolated and washed with 0.5 *M* sucrose so that the cytoplasm was completely removed before exposure to 30 per cent Carbowax 4000. Their storage deterioration rates in Carbowax vs. sucrose were now the same. The negligible preservative action of polyethylene glycols upon stored chloroplasts observed by Bishop, Lumry and Spikes (1) referred to chloroplasts which had been similarly freed of cytoplasm.

Measurements of alfalfa chloroplast deterioration *in vivo* at 0° C. (within intact, attached leaves) indicated better retention of photochemical activity than during storage in Carbowax-cytoplasm. Barring metabolic complications, chloroplast deterioration in Carbowax-cytoplasm is intermediate to that occurring in living cells and in 0.5 *M* sucrose. The rapid loss of Hill reaction activity from chloroplasts in whole spinach plants (70—90 per cent loss in 4 days) reported by Clendenning and Gorham (4) referred to dark storage at ca. 25° C.

The stabilizing action of cytoplasm upon chloroplasts in Carbowax at 0° C., and in sucrose at —40° C. (7) apparently is also operative during the frozen storage of intact leaves. When Gleditsia leaves are packaged in sealed envelopes and stored at —20° C., their chloroplasts' capacity for the Hill reaction *in vitro* remains constant for weeks.

(c) *Temperature relations*

Hill reaction capacities of alfalfa chloroplasts were determined over a range of temperatures in 30 per cent Carbowax 4000 and 0.5 *M* sucrose. In one series (Figure 3), alfalfa leaves were ground in 30 per cent Carbowax 4000, and the chloroplast-cytoplasm sediments were taken up in (a) 30 per cent Carbowax (b) 0.5 *M* sucrose. As in Figure 1, these chloroplast suspensions contained equal amounts of cytoplasm, precipitated on the plastids in the Carbowax, and uniformly dissolved in the sucrose solution (Figure 3). Leaf cytoplasm was excluded in a second temperature series by reversing the chloroplast isolation procedure: the leaves were ground in 0.5 *M*

Figure 3. *Temperature relations of water photolysis capacity in alfalfa chloroplasts isolated in 30 per cent Carbowax 4000 and resuspended in Carbowax and sucrose solution.*

o - - - - o 30 per cent Carbowax 4000
△————△ 0.5 M. sucrose
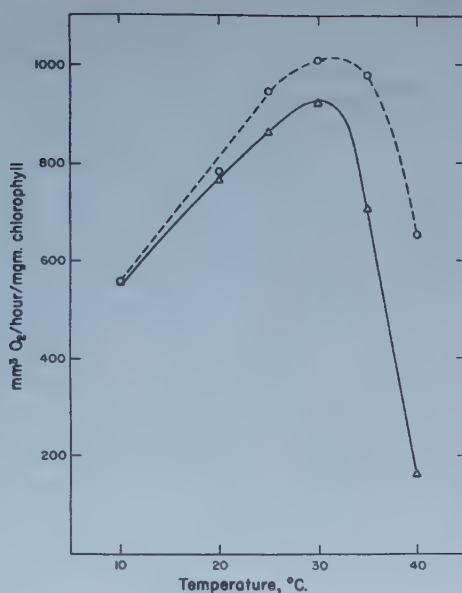
sucrose and the resulting chloroplast suspensions were centrifuged, washed with fresh 0.5 M sucrose, and recentrifuged. The supernate was again discarded and the sediments were taken up in 30 per cent Carbowax and 0.5 M sucrose (Figure 4). The chlorophyll concentration was uniform (0.2 mg. chlorophyll per vessel); aliquots of the chloroplast suspensions were withdrawn successively and subjected to the quinone for fifteen minutes and to the bath temperature for thirty minutes prior to illumination for twenty minutes. The stock suspensions were maintained at 0° C., and a correction was introduced for storage deterioration at 0° C. during the experiment, which was based on successive activity measurements at a standard temperature. The lower activity levels of Figure 3 (vs. Figure 4) were caused by the use of chloroplasts which were stored for one day at 0° C. in 30 per cent Carbowax 4000. The features of Figure 3 were confirmed in subsequent experiments involving fewer temperatures.

Under the foregoing conditions, the optimum temperature for water photolysis in chloroplast-cytoplasm (Figure 3) was 30° C. in both media. Above this temperature, the stabilizing action of the Carbowax-cytoplasm became increasingly apparent. At 40° C., the alfalfa chloroplasts in 0.5 M sucrose were rapidly inactivated during the 20-minute activity measurements, in contrast to the chloroplast-cytoplasm of the Carbowax suspensions which exhibited activity quotients of > 600 at 40° C.

The effects of temperature upon the Hill reaction activities of chloroplasts suspended in 30 per cent Carbowax and 0.5 M sucrose in the absence of cytoplasm were essentially identical; the »optimum» temperature was 25° C. in both cases, and thermal inactivation at higher temperatures proceeded
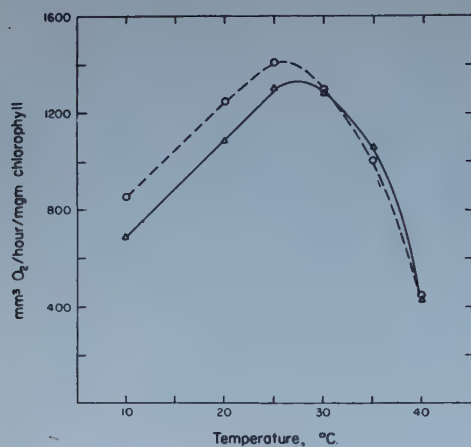
Figure 4. *Temperature relations of water photolysis capacity in alfalfa chloroplasts isolated and washed in 0.5 M sucrose, then resuspended in Carbowax and sucrose.*
○ - - - -○ 30 per cent Carbowax 4000
△————△ 0.5 M. sucrose

at equal rates (Figure 4). The stabilizing action of Carbowax 4000 upon water photolysis at high temperatures as well as upon storage deterioration at 0° C. is therefore exerted indirectly by way of the cytoplasm.

Discussion

The present study revealed two effects of 30 per cent Carbowax 4000 upon leaf macerates which elucidate its indirect action on water photolysis *in vitro*. It exerts a mild »salting-out» action on the cytoplasmic proteins so that the chloroplasts remain embedded in cytoplasm after isolation. The stabilizing action of Carbowax on isolated chloroplasts must be exerted by way of the cytoplasm since it is not evident when the chloroplasts are freed of cytoplasm beforehand. This effect is independent of tannins; it becomes increasingly important at high reaction temperatures and during long periods of storage. The physical or chemical causes of chloroplast deterioration *in vitro* and of stabilization *in vivo* and *in vitro* by cytoplasm remain unknown.

The second effect of the Carbowax is exerted by way of the leaf tannins, which otherwise cause chloroplast inactivation. The affinity of Carbowax for tannins in solution lessens their adsorption by chloroplasts as well as by Collagen, and also blocks their extraction from water with ethyl acetate. If the chloroplasts are not inactivated before their release from tannin-containing cells, their Hill reaction activity is increased through this Carbowax-tannin interaction. Several examples are cited of leaves which yielded active chloroplasts for this reason when isolated with 30 per cent Carbowax 4000 but not with 0.5 *M* sucrose.

Summary

When leaf chloroplasts are extracted in 30 per cent Carbowax 4000, cytoplasm remains with the plastids and their capacity for the Hill reaction is stabilized. The stabilizing action of Carbowax on chloroplasts is exerted indirectly through the cytoplasm. The initial capacity for water photolysis is higher in Carbowax-cytoplasm when the activity measurements are made under chloroplast-limited conditions. The stabilizing effect of the condensed cytoplasm becomes increasingly evident at high temperatures and after long storage periods. The inactivation of chloroplasts by leaf tannins during the isolation procedure is impeded by Carbowax through its affinity for tannin in solution.

Note added in proof: C. R. Stocking (Science 123: 1032, 1956) has reported that catalase and phosphorylase are precipitated on the chloroplasts when concentrated Carbowax solutions are employed as leaf-grinding fluids. His findings support our conclusions.

References

1. Bishop, N. I., Lumry, R. & Spikes, J. D.: The mechanism of the photochemical activity of isolated chloroplasts. 1. Effect of temperature. — Arch. Biochem. Biophys. 58: 1. 1955.
2. Clendenning, K. A. & Ehrmantraut, H. C.: Photosynthesis and Hill reactions by whole Chlorella cells in continuous and flashing light. — Arch. Biochem. 29: 387, 1950.
3. — & Gorham, P. R.: Photochemical activity of isolated spinach chloroplasts in relation to reaction conditions. — Can. J. Research C 28: 78, 1950.
4. — — Photochemical activity of isolated chloroplasts in relation to their source and previous history. — Can. J. Research C 28: 114, 1950.
5. — Brown, T. E. & Walldov, E. E.: Natural inhibitors of the Hill reaction. — Proceedings of the Second International Conference on Photosynthesis, Gatlinburg, Tenn., 1955 (in press).
6. Crafts, A. S., Currier, H. B. & Stocking, C. R.: Water in the physiology of plants. — Chronica Botanica Co., Waltham, Mass., 1949.
7. Gorham, P. R. & Clendenning, K. A.: Storage of isolated chloroplasts without loss of photochemical activity. — Can. J. Research C 28: 513, 1950.
8. Haxo, F. T., O'h Eocha, C. & Norris, P.: Comparative studies of chromatographically separated phycoerythrins and phycocyanins. — Arch. Biochem. Biophys. 58: 162, 1955.
9. MacKinney, G.: Absorption of light by chlorophyll solutions. — J. Biol. Chem. 140: 315, 1941.
10. McClendon, J. H. & Blinks, L. R.: Use of high molecular weight solutes in the study of isolated intracellular structures. — Nature 170: 577, 1952.
11. — The physical environment of chloroplasts as related to their morphology and activity in vitro. — Plant Physiol. 29: 448, 1954.
12. Meyer, B. S. & Anderson, D. B.: Plant Physiology. — D. Van Nostrand Co., Inc., New York, 1939.

13. Official and Tentative Methods of Analysis. Association of Official Agricultural Chemists. — Washington, D. C., 6th Ed., 1945.

14. Shiroya, M. & Hattori, S.: Studies on the browning and blackening of plant tissues. — Physiol. Plant. 8: 358, 1955.

15. Takada, H.: The tannin-conveying idioblasts in the leaf lamina of Elodea densa. — J. Inst. Polytech., Osaka City University Ser. D, 31, 1952. (Chem. Abstracts 48: 10135 a, 1954.)

16. Thomas, J. B. & De Rover, W.: On phycocyanin participation in the Hill reaction of the blue-green alga Synechococcus cedrorum. — Biochim. et Biophys. Acta 16: 391, 1955.

17. Uritani, I., Hoshiya, I. & Takita, S.: Phytopathological chemistry of black-rotted sweet potato XI. — J. Agric. Chem. Soc. Japan 27: 785, 1953. (Chem. Abstracts 48: 12934 g, 1954.)

18. Waygood, E. R., & Clendenning, K. A.: Intracellular localization and distribution of carbonic anhydrase in plants. — Science 113: 177, 1951.

The Relation between Nitrogen Assimilation and Respiration in *Scopulariopsis brevicaulis*

By

ANNE MACMILLAN

Imperial Chemical Industries Ltd., Akers Research
Laboratories, Welwyn, Herts.
(Received May 25, 1956)

Introduction

A close relationship exists between nitrogen assimilation and respiration. Thus in yeast (Yemm and Folkes, 1954) and in Chlorella (Syrett, 1953) rapid ammonia assimilation can occur in the absence of added sugar, and the assimilation is linked with an increase in endogenous respiration. In the fungus *Scopulariopsis brevicaulis* it was found (Morton and MacMillan, 1954) that stored reserves do not support nitrogen assimilation and a supply of glucose is required for assimilation. The present work examines the relation between nitrogen assimilation and respiration in *S. brevicaulis* in an attempt to understand why, in this organism, ammonia is not assimilated during endogenous respiration.

The results have shown that added glucose serves both as energy and carbon source for assimilation. Without glucose, protein synthesis does not take place even though sufficient substrates are available to support prolonged endogenous respiration as well as an initial synthesis of amino acids.

Experimental Methods

Scopulariopsis brevicaulis Sacc. (Bain.) was used and was grown as described by MacMillan (1956) by shake culture in a synthetic medium. The even suspension of mycelium was harvested after slightly less than 3 days' growth, and washed 2—3 times with distilled water before use.

[533]

Nitrogen assimilation was determined as described by Morton and MacMillan (1954), the loss of ammonia or nitrate from the suspension of mycelium being determined per unit dry weight of mycelium. Differences of less than 1 mg. N/g. dry weight are not significant. — *Respiration rates.* Conventional Warburg techniques were used and aliquots of a suspension of mycelium in phosphate buffer measured into the flasks. Where cyanide was present the method of Robbie (1948) was followed. — *Soluble nitrogen* in the mycelium was extracted in the cold (4° C) with 65 per cent v/v ethanol after addition of absolute alcohol equivalent to twice the volume of the mycelium. The extract was filtered after 20 hr., the residue washed with 65 per cent ethanol and the filtrate made to a suitable volume. Total nitrogen and amino-nitrogen were determined in aliquots of the extract. For *chromatograms* the alcohol extract was allowed to stand with 4—5 times its volume of chloroform (Block, Durrum and Zweig, 1955) and aliquots of the aqueous layer which contains the amino acids were run with butanol-acetic acid-water and with phenol as developers and sprayed with ninhydrin. — *Total nitrogen* was determined by Kjeldahl method using selenium as catalyst for the digestion and 2 per cent w/v boric acid as absorbent for the distillation. — *Ammonia and nitrate* estimation was by the method of Conway and Byrne (1933) using boric acid as absorbent and 0.5 N KOH to liberate the ammonia. Nitrate was reduced to ammonia in the units by addition of Devarda's alloy. In mycelial extracts the vacuum distillation method described by Koch and Hanke (1948), which does not hydrolyse amides, was used. Nitrate was first reduced to ammonia by standing 20 hr. with iron and 2 N H_2SO_4, blanks without iron being run simultaneously. — *Amino-nitrogen* was determined after reaction with ninhydrin (Sobel *et al.*, 1945) the ammonia being liberated by vacuum distillation as described by Koch and Hanke (1948) and Nesslerised. — *Glucose and carbohydrate* were estimated by the anthrone method as modified by Fairbairn (1953). Aliquots of a suspension of finely ground dried mycelium were used. — *Fatty acid content* of the mycelium is expressed as weight of material obtained by exhaustive ether extraction of dried mycelium previously hydrolysed 2 hours with N HCl.

Experimental Results

Assimilation of Ammonia and Nitrate

For the experiments the mycelium was used after a little less than 3 days' growth. The nitrogen source of the culture medium is exhausted after about $2\frac{1}{2}$ days', so that the mycelium is deprived of nitrogen for some hours. When such mycelium is placed in phosphate buffer containing an ammonium salt $[(NH_4)_2SO_4]$ and shaken to provide oxygen, ammonia is assimilated at a rate of 3.5—4.0 mg. N/g. D.Wt./hr. (Morton and MacMillan, 1954). If glucose is supplied the assimilation proceeds for at least 7 hours, but in its absence stops after 1—2 hr. (Figure 1). The assimilation results in an initial increase in the soluble nitrogen of the mycelium, and most of this increase is in the form of α-amino-nitrogen (Table 1). Protein is formed during more
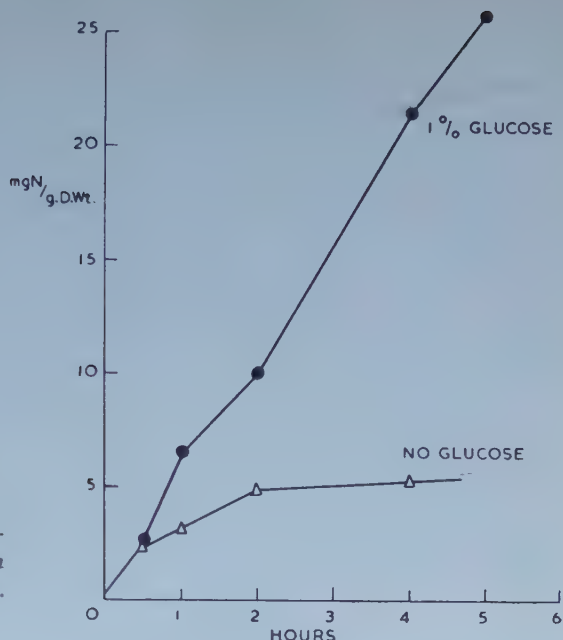
Figure 1. *The assimilation of ammonia by S. brevicaulis from ammonium sulphate solution* (pH 7, 0.4 mg. N/ml.).

prolonged assimilation with glucose, but in its absence very little, if any, protein synthesis occurs.

Examination of the alcohol-soluble nitrogen fraction by paper chromatography (Table 2) showed that during the initial assimilation there is a big increase in the amount of glutamine present. This is followed by a similar increase in alanine and glutamic acid. Smaller increases occur in the levels of other acids, especially proline. Asparagine was found hardly to increase.

Table 1. *The uptake of ammonia and the products of ammonia assimilation in S. brevicaulis, shaken in phosphate buffer (as mg. N/g. D.Wt.).*

Time	NH₃ uptake	Soluble N present	Protein formed (by difference)	NH₂−N present
		with glucose		
Initial	—	6.2	—	1.5
1 hr.	6.0	9.9	—	3.5
2 hr.	11.2	9.2	—	4.7
5 hr.	23.6	10.1	20	4.9
		without glucose		
Initial	—	6.1	—	1.6
1 hr.	4.1	10.0	—	3.9
2 hr.	5.1	10.8	—	5.1
5 hr.	5.3	10.5	0—2	5.0
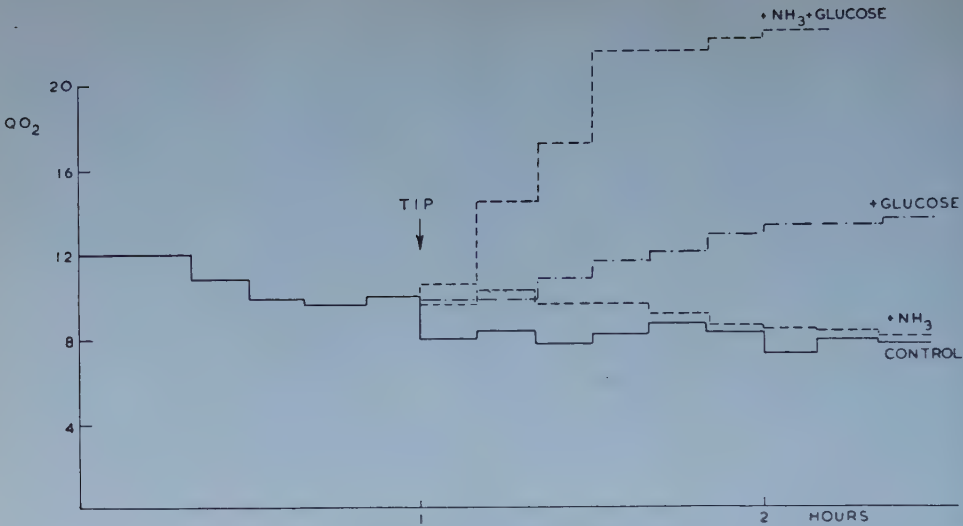
Figure 2. *Oxygen uptake by mycelium of S. brevicaulis and effect of glucose* (1 % w/v) *and ammonium sulphate* (0.4 mg. N/ml.).

There is no apparent difference in the soluble nitrogen compounds formed by ammonia assimilation in the presence or absence of glucose. If nitrate (as KNO_3) is supplied instead of ammonia, similar results are obtained, but the rate of assimilation is less. The amino acids formed from nitrate are also similar.

Since nitrogen assimilation requires respiratory energy, it was thought that the short duration of assimilation without glucose could be due to a rapid decline in the endogenous respiration rate. The respiration rates during assimilation were therefore investigated. Figure 2 shows the endogenous respiration of the mycelium after it is filtered from the culture medium, washed and placed in phosphate buffer at pH 7. The QO_2 falls off slightly

Table 2. *Formation of amino acids by mycelium of S. brevicaulis during assimilation of ammonia in the presence or absence of glucose.*

Amino acid	Initial	1 hr.	2—5 hr.
Alanine	+	+	++++
Glutamic	+	+++	++++
Glutamine	+	++++	++++
Aspartic	±	±	+
Asparagine	+	+	++
Proline	±	+	++
Glycine	±	±	+
Serine	±	±	+
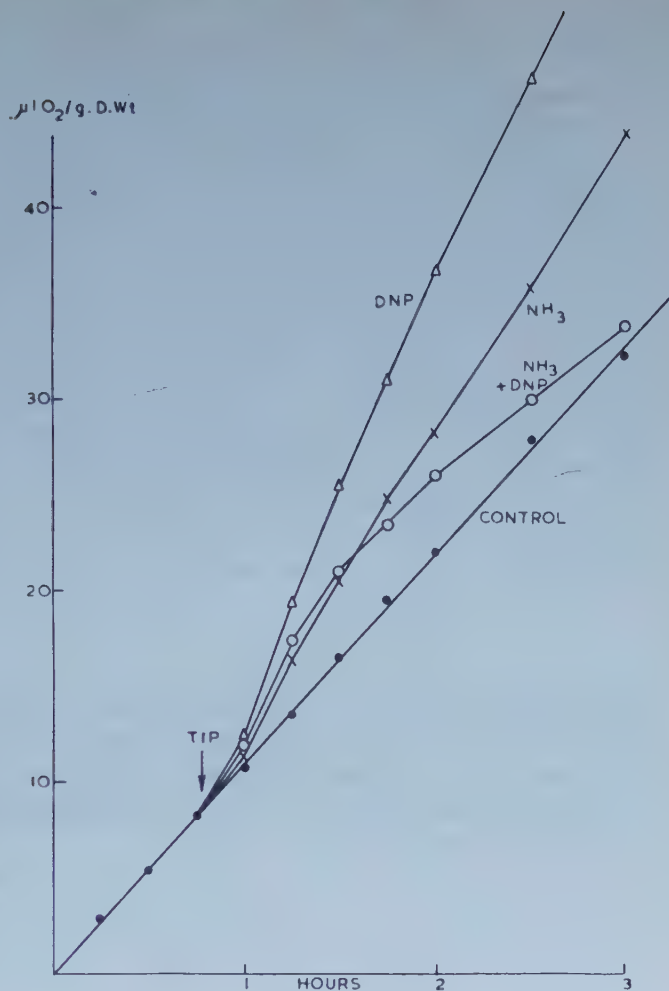
Figure 3. *Effect of DNP (2.5×10⁻³ M) on oxygen uptake rate of S. brevicaulis with and without ammonium sulphate (0.4 mg. N/ml.).*

during the first 1—2 hr. (usually by about 15 per cent) and is subsequently maintained at a fairly constant rate for several hours. If ammonia is added, there is a small rise in QO_2 lasting about 1 hour and this probably corresponds to the period of ammonia assimilation. However, no marked fall in respiration rate occurs, which might account for the failure of assimilation to continue after this time.

If glucose is supplied, the oxygen uptake rate rises for about 2 hr. to a new level, 25—50 per cent above the endogenous rate. If ammonia is supplied together with glucose the increase in QO_2 is even greater and this is undoubtedly connected with the protein synthesis which occurs. If nitrate is supplied instead of ammonia, the results again are very similar.
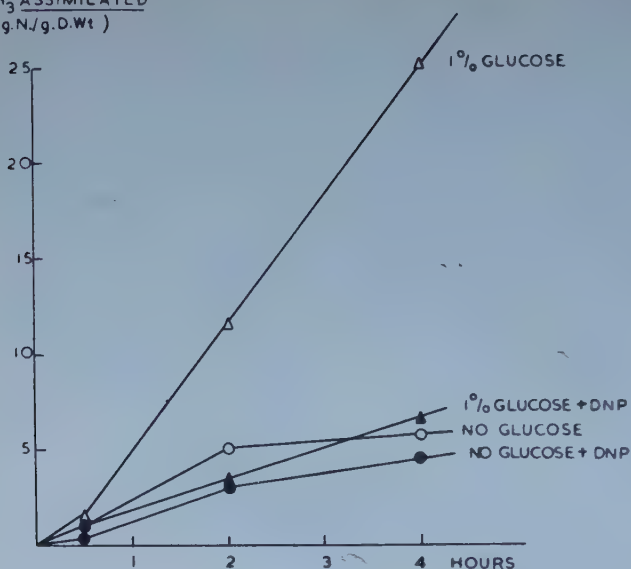
Figure 4. *Effect of DNP $(2.5 \times 10^{-3} M)$ on assimilation of ammonia by S. brevicaulis with and without glucose (at pH 7).*

The endogenous oxygen uptake is not inhibited by low concentrations of 2:4-dinitrophenol (DNP) and may be increased (Figure 3). The increased respiration due to ammonia is, however, inhibited by DNP and so also is the assimilation of ammonia (Figure 4). This is consistent with the view that DNP uncouples oxidation from phosphorylation, so that energy required for assimilation cannot be transferred.

An interesting effect of DNP is that the initial formation of soluble nitrogen is not inhibited but that the amino acids formed are different. In the presence of DNP the ammonia assimilated is found largely as alanine, only smaller amounts of glutamine and other amino acids being formed (Table 3).

Table 3. *The effect of DNP $(2.5 \times 10^{-3} M)$ on amino acids in soluble nitrogen fraction after 2 hr. in ammonium sulphate (0.4 mg. N/ml.) at pH 7.*

Amino acid	1 % glucose		No glucose	
	Control	DNP	Control	DNP
Alanine	+ + + +	+ + + + + +	+ + +	+ + + + + +
Glutamic	+ + + +	+ +	+ + + +	+ +
Glutamine	+ + +	+	+ + +	+
Aspartic	+ +	±	+ +	±
Asparagine	+	—	+	—
Proline	+ + +	+	+ + +	+
Total NH$_2$-N present ...... (as mg./g. D.Wt.)	5.4	6.8	5.9	6.6

Utilisation of Endogenous Reserves

In *S. brevicaulis* nitrogen assimilation is accompanied by a rise in QO_2, but this increase like the assimilation is maintained only if an exogenous energy source is available. However, it is not clear why, if the endogenous respiration supports ammonia assimilation for 1—2 hr. the assimilation should stop after this time, when the respiration rate has hardly changed. Moreover, experiments described below show that the mycelium contains reserve material sufficient to maintain endogenous respiration for many hours. Samples of mycelium were "starved" by shaking for 24 hr. and longer in phosphate buffer. Figure 5 shows that after 24 hr. treatment the endogenous respiration rate has dropped to about half the initial value, but ammonia is assimilated by this starved mycelium for about 1 hr., (Figure 6) and this is accompanied by a rise in respiration rate (Figure 5). In the presence of glucose the starved mycelium assimilates ammonia somewhat slower than the unstarved material, and there is a smaller rise in respiration. After longer periods of starvation the rates of endogenous respiration and ammonia assimilation become much slower, so that after 3 days the QO_2 is 15 per cent of the initial rate. Addition of glucose, however, now produces a five-fold increase in QO_2.

There is thus sufficient respiratory substrate to maintain an endogenous respiration for over 24 hr., albeit at a reduced rate, but its oxidation does not support nitrogen assimilation. This is further shown by the carbon balance sheet comparing endogenous and glucose respiration (Table 4).
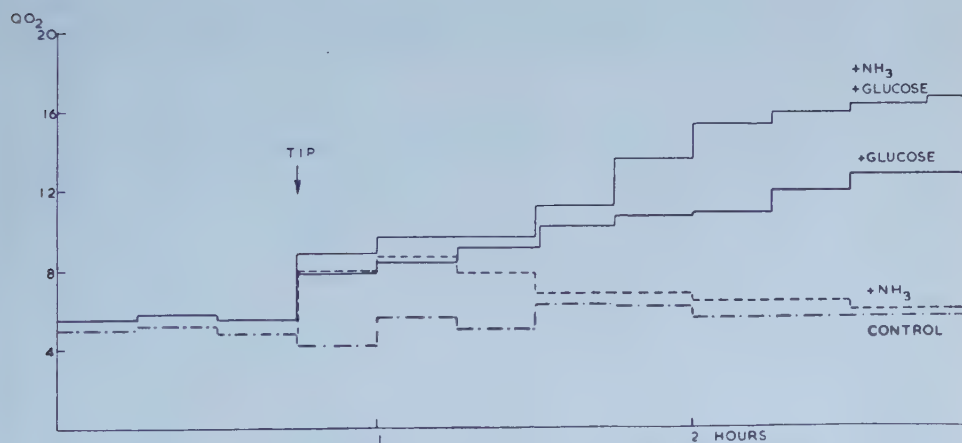
Figure 5. *Oxygen uptake by mycelium of S. brevicaulis previously starved 24 hr. in phosphate buffer.* Glucose (1 %/o w/v) and ammonium sulphate (0.4 mg. N/ml.) added where shown.
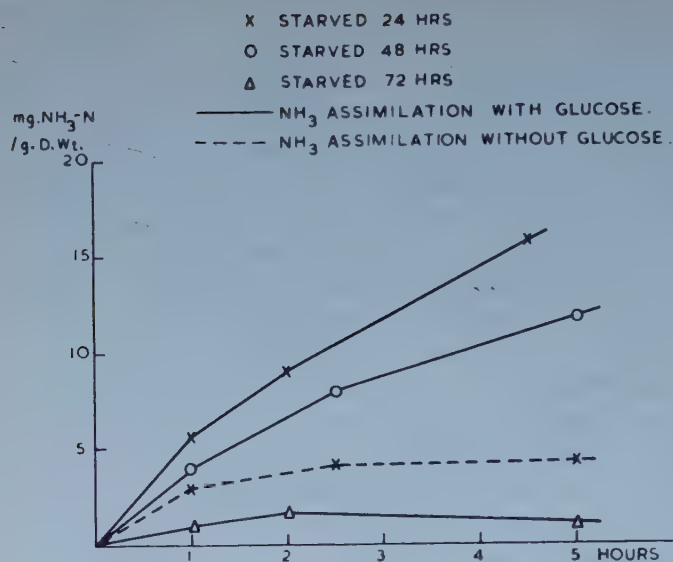
Figure 6. *Ammonia assimilation by mycelium of S. brevicaulis previously starved for different periods in phosphate buffer.*

During a 5 hr. period the loss in dry weight due to endogenous respiration is accounted for by the CO_2 evolved. In addition a small quantity of endogenous material is metabolised in the synthesis of amino acids from ammonia. When glucose is supplied, this is utilised at a much faster rate than the endogenous substrates. Half the glucose utilised is incorporated into the protein formed and this is reflected in the increased dry weight. Rather less than half the glucose used is oxidised to CO_2 in this case, though in some experiments a higher rate of CO_2 evolution was observed.

It is clear from these figures that the endogenous reserves could not for

Table 4. *Carbon balance sheet for S. brevicaulis during ammonia assimilation with and without glucose supply (as mg. C/g. D.Wt./5 hr.).*

Data estimated	Endogenous	With 1% glucose
RQ	0.6	0.9—1.0
Glucose utilised	—	150
CO_2 liberated	18	55
Dry weight loss or gain	—16—20	+80
Carbohydrate loss	4—7	—
Fatty acid loss	2—6	—
Protein loss (Total N)	0	—
Dry weight loss in 24 hr. ..... (to indicate reserves available)	80—100	
NH_3 assimilated ..... (as carbon in amino-acids formed)	6—10	70

long maintain respiration and ammonia assimilation at the rate at which they proceed with glucose. On the other hand, during 5 hours' endogenous respiration, only a small part of the total available reserves are metabolised if we take the dry weight loss over 24 hr. as a guide to the amount available. It is of interest, therefore, to examine why some of this reserve is not mobilised to support more prolonged ammonia assimilation.

The difference between the respiration of endogenous reserves and that with glucose lies in the nature of the substrate, since the endogenous reserves clearly cannot be in the form of glucose. The RQ when glucose is supplied is 0.9—1.0. That of the endogenous respiration is much lower (0.6) suggesting that fats may be used. The preliminary examination that has been made so far shows that although the mycelium contains about 10 per cent fatty acid (on a dry weight basis) only about 5—10 per cent of this is used during 5 hours' endogenous respiration, and this accounts for only a small part of the total substrate respired. Rather more carbohydrate is used, but the observed fat and carbohydrate utilisation accounts for only half the dry weight loss. Further studies of the endogenous substrates are required to clarify this discrepancy. Protein is apparently not used as there is no loss in total nitrogen from the mycelium.

Another difference between the endogenous and the glucose respiration lies in the rate at which they proceed and in the fact that while the rate of glucose respiration is further increased by the addition of ammonia, the endogenous rate is hardly affected. Although the latter difference may result indirectly from ammonia assimilation it shows that the rate at which endogenous reserves are mobilised is not readily increased.

It was also thought possible that the initial synthesis of amino acids is at the expense of an organic acid pool formed during previous growth on glucose medium. Once this is used up in the formation of amino acids, endogenous respiration might be unable to replenish it so that assimilation stops.

To test this possibility a large number of organic acids and other short chain compounds were examined for their ability to promote ammonia assimilation. None of 15 substances tried (at 1 per cent w/v) produced any increase in the amount of ammonia assimilated, even though some (e.g. acetate, glycerol) are used as respiratory substrates. It is unlikely, therefore, that failure to enter the cell is the cause of the negative results. Only hexoses, such as mannose and fructose have been found to replace glucose in promoting assimilation.

In an attempt to increase the rate and duration of endogenous respiration "nitrogen starved" mycelium was prepared by treatment with a carbohydrate supply in the absence of a nitrogen source. A 3-day old culture was centrifuged and the medium replaced aseptically by fresh medium complete except

Table 5. *Oxygen uptake and ammonia assimilation by "low nitrogen" mycelium (i.e. 3-day old mycelium shaken 24 hr. in nitrogen-free culture medium.*

Data estimated	Control mycelium		»Low-N» mycelium
	3 days	4 days	
Total N content (mg. N/g. D.Wt.)			
Initial	66		
5 hr.	—	—	56
24 hr.	—	53	49
Ammonia assimilation (mg. N/g. D.Wt./hr.)			
No glucose	3— 4[1]	—	2— 3[1]
With glucose	4— 5	—	2— 3
QO_2			
No glucose −NH_3	11	7	5— 6
+NH_3	12	—	8
With glucose −NH_3	14—15	—	9—12
+NH_3	18—22	12	13—18

[1] For one hour only.

for the nitrogen source. After shaking for 24 hr. the respiration and nitrogen assimilation rates were compared with the initial rates and the rates of control mycelium shaken a further 24 hr. in the original medium. Table 5 shows the results of this experiment and of others in which the mycelium was shaken in phosphate buffer containing glucose. Non-nitrogenous reserves are increased by this treatment and the proportion of nitrogen in the mycelium falls. However, the endogenous respiration rate is about one half of the original rate. The addition of ammonia now produces a larger increase in respiration rate, though the rate is still less than initially. If glucose is supplied, ammonia assimilation is also slower than initially. Thus mycelium with larger carbon reserves appears to be metabolically less active than the untreated 3-day old mycelium.

In this respect nitrogen starvation is similar to the normal development of the mycelium in our culture media, from which the nitrogen source, but not the glucose, is exhausted after 2 1/2 days' growth. After this time the mycelium accumulates non-nitrogenous reserves and the respiration rate falls (Table 5). This process continues until after 5 days' growth the dry weight is almost double that at 3 days, but there is no longer a measurable endogenous respiration. Autolysis does not become apparent until several days later.

Discussion

When mycelium of *S. brevicaulis*, previously growing for a short period without a nitrogen source, is supplied with ammonia or with nitrate, assi-

milation occurs and results in a new higher level of amino-acids in the cells. These are mainly glutamine, which appears to be formed first, and glutamic acid and alanine. Similar results have been described by other workers using Yeast (Roine, 1947; Yemm and Folkes, 1954) and *Chlorella*, (Syrett, 1953). The increase in amino acids is followed by protein synthesis, but in *S. brevicaulis* appreciable protein synthesis occurs only if glucose is supplied. Endogenous reserves do not support continued ammonia assimilation. In this respect *S. brevicaulis* differs from the organisms mentioned above.

It appears that the available endogenous reserves are unsuitable to support protein synthesis. Present evidence has shown these to consist at least in part of fat and carbohydrate (anthrone-reacting), but further analysis is required. The factor limiting further assimilation is not apparently the supply of short-chain carbon compounds (e.g. organic acids) for combination with ammonia, first because the initial synthesis of amino acids is as rapid without glucose as in its presence, and secondly because addition of a variety of suitable compounds does not promote continued assimilation.

Although ammonia assimilation is accompanied by a rise in respiration rate, it is possible that the breakdown of endogenous substrates does not release sufficient energy in the form of high energy phosphate for protein synthesis. On the other hand, the fact that glutamine is rapidly formed suggests that an adequate supply of adenosine triphosphate (ATP) is available at any rate initially, since it is required for the enzymic amide bond formation of glutamine (Elliot 1951).

In this connection the results with dinitrophenol (DNP) are interesting, because although this does not inhibit oxygen uptake, it is thought to inhibit formation of ATP. In *S. brevicaulis* the initial synthesis of glutamine is inhibited by DNP. The alanine formed in its place could arise without the intervention of ATP by reductive amination of pyruvic acid. DNP also prevents the increase in QO_2 due to ammonia assimilation in *S. brevicaulis* and this may be linked with the inhibition of glutamine and protein synthesis. This would be in accordance with the proposals of Yemm and Folkes (1954) and Syrett (1953) that the increase in QO_2 accompanying ammonia assimilation is due to reduction in the level of ATP caused by glutamine synthesis.

The inability of the endogenous respiration to maintain ammonia assimilation is not due to an inadequate enzyme complement since, in the presence of glucose, assimilation starts at the maximum rate. Nor is it due to the rapid depletion of endogenous reserves. Endogenous respiration continues at a fairly constant rate for many hours, and only a small fraction of the endogenous oxidisable materials is utilised during the initial period of ammonia assimilation. Moreover, the experiments with "low nitrogen"

mycelium prepared by sugar feeding, showed that when the supply of endogenous substrates is increased the rate at which they are used tends to decrease. This is undoubtedly connected with the fact that the various treatments produce cells of greater age. In *S. brevicaulis* older cells which have been deprived of nitrogen respire more slowly, in spite of the much greater carbon reserves they contain. This may be due to a falling off in enzyme activity in cells which are not assimilating nitrogen. It is also possible that while the enzymes concerned with ammonia assimilation are most active at growing hyphal tips, the bulk of the endogenous carbon reserves may be located in the older and metabolically less active parts and not readily available for nitrogen assimilation. Such a spatial separation could account for the difference between *S. brevicaulis* and the unicellular organisms, *Chlorella* and yeast, whose cells readily utilise endogenous carbon reserves for protein synthesis.

Summary

When ammonia or nitrate is supplied together with glucose to the young mycelium of *Scopulariopsis brevicaulis* there is an initial synthesis of soluble nitrogen compounds. This is followed by protein synthesis which continues for many hours and is accompanied by an increase in respiration rate.

In the absence of glucose, the initial synthesis of amino acids occurs but nitrogen assimilation stops after a short period, although endogenous respiration continues at a slowly decreasing rate for many hours, and only a small part of the endogenous respiratory substrates have been utilised when nitrogen assimilation ceases. In "nitrogen starved" mycelium, which contains greater carbon reserves, assimilation also requires added glucose. The results show that the endogenous reserves are less readily mobilised for respiration and nitrogen assimilation than externally supplied glucose.

References

Block, R. J., Durrum, E. L. & Zweig, G.: A manual of paper chromatography and paper electrophoresis. — Acad. Press, New York, p. 84. 1955.

Conway, E. J. & Byrne, A.: An absorption apparatus for the microdetermination of ammonia. — Biochem. J., 27: 419. 1933.

Elliot, W. H.: Studies in the enzymic synthesis of Glutamine. — Biochem. J., 49: 106. 1951.

Fairbairn, N. J.: A modified anthrone reagent. — Chem. & Ind., p. 86. 1953.

Koch, F. C. & Hanke, M. E.: Practical methods in biochemistry. — Williams & Wilkins Co., Baltimore. 1948.

MacMillan, Anne: The entry of ammonia into fungal cells. — J. Exp. Bot. 7: 113. 1956.

Morton, A. G. & MacMillan, Anne: The assimilation of nitrogen from ammonium salts and nitrate by fungi. — J. Exp. Bot., 5: 232. 1954.

Robbie, W. A.: Use of cyanide in tissue respiration studies. — Methods in Medical Research, 1: 307. 1948.

Roine, P.: On the formation of primary amino acids in the protein synthesis in yeast. — Ann. Acad. Sci., Fennicae, Ser. A., 26: 1947.

Sobel, A. E., Hirschmann, A. & Besman, Lottie: A convenient microtitration method for the estimation of amino acids. — J. Biol. Chem., 161: 99, 1945.

Syrett, P. J.: The assimilation of ammonia by nitrogen-starved cells of Chlorella vulgaris. — Ann. Bot. N.S., 17: 1. 1953.

Yemm, E. W. & Folkes, B. F.: The regulation of respiration during the assimilation of nitrogen in Torulopsis utilis. — Biochem. J., 57: 495. 1954.

Selective Inhibition of the Geotropic Response by n-1-Naphthylphthalamic Acid[1]

By

Te-May Tsou Ching, Robert H. Hamilton[2] and Robert S. Bandurski

Department of Botany and Plant Pathology Michigan State University,
East Lansing, Michigan, U.S.A.
(Received June 1, 1956)

Introduction

According to the theory of Went and Cholodny (1937), the geotropic response of plant seedlings is due to differential growth following an internal redistribution of auxin. Since geotropic curvature results simply from unequal growth, a direct correlation between growth rate and rate of geotropic curvature is to be expected and an inhibition of growth should result in a proportional inhibition of curvature. In previous studies from this laboratory (Grigsby et al, 1954) it was found that treatment of pea seedlings with n-1-naphthylphthalamic acid (NP) resulted in a 70 percent reduction of straight growth and a complete loss of geotropic sensitivity. Nétien and Conillot (1951) had earlier made a similar, although qualitative, observation. This disproportionality of growth and geotropic response, if significant, would seem to require a modification of the classical theory of the tropic mechanism. A study was therefore initiated to examine these observations quantitatively and to extand them to several additional plant species.

Hoffmann and Smith (1949) have observed growth regulating effects of derivatives of phthalamic acid. They stated that at 0.1 ppm, NP induced

leaf-rolling in tomato plants; at 0.31 ppm, epinasty was observed; and at 20 ppm, stem swelling. Mentzer, Molho, and Pacheco (1950) tested twenty-five related compounds on lentil roots, and concluded that a close spatial relationship of the peptide bond and a carboxyl group was required for inhibition of geotropism. Mentzer and Nétien (1950) studied the effect of NP on the geotropic response of pea, lentil, crucifer, tomato, sunflower, and cucumber seedlings, and found that 10^{-3} to 10^{-6} M concentrations caused an apparent negative geotropism. Treated tap roots were somewhat thicker and shorter than the control. In a study of the effect of NP on germination and early stages of growth, Nétien and Conillot (1951) claimed that the chemical retarded the germination of some species and inhibited straight growth in most species tested.

Materials and methods

Commercial grade NP was recrystallized from acetone-petroleum ether. For use, the recrystallized material (m.p. 191°) was converted to the potassium salt by the addition of a stoichiometric amount of potassium bicarbonate in aqueous-acetone solution. The salt was recovered by evaporation of the solvent in vacuo at 37° C with ebullition of dry nitrogen gas. Spectophotometric analysis yielded a molar extinction coefficient of $E_{284} = 1.595 \times 10^4$.

The standard Avena coleoptile section test (McRae, Foster and Bonner, 1953) was used to determine the interaction of indoleacetic acid (IAA) and NP. The standard error of each test was less than 10 percent, and there was no significant difference between replicates.

For straight growth and geotropic curvature tests, intact seedlings were used. Victory oats (*Avena sativa* var. Siegeshafer) were obtained through the courtesy of Dr. J. Bonner. Hybrid corn (*Zea mays*, W23×Oh51a, a single cross hybrid) was kindly supplied by Dr. E. Rossman, Department of Farm Crops, Michigan State University. Garden pea (*Pisum sativum* var. Alaska) was obtained from the Ferry Morse Seed Company.

Seeds were germinated in the dark at 25° C and all subsequent operations were conducted in subdued red light. Seedlings with straight tap roots and shoots of a desired length were selected for the experiments. Avena coleoptiles and roots of corn and pea were harvested at 2.0 ± 0.5 cm. while shoots of corn and pea were used at 1.25 ± 0.25 cm. Seedlings were pretreated with NP or other compounds by immersion of the seedlings in a solution of the compound dissolved in 0.005 to 0.01 molar phosphate buffer at pH 4.5. Control plants were similarly treated in the buffer. Pre-treatment time for oat coleoptiles was 30 minutes and 60 minutes for both roots and shoots of corn and pea. A longer pre-treatment time was used for corn and pea seedlings since the response subsequent to 30 minute treatment was not uniform. After pre-treatment the seedlings were divided into two groups; one for the geotropic test, and the other for the straight growth test. For the geotropic test, plants were held in a horizontal position by means of an Avena holder or by means of pressure sensitive tape with adhesive on both sides. The tape was mounted

on a moist blotter paper and the seeds covered with moist tissue paper. For the straight growth test, plants were held in a vertical position by the same means. Both sets were incubated at 25° C and 90 percent relative humidity.

Geotropic curvature was measured from shadowgraphs. Ten to sixteen plants were used per treatment and each experiment repeated one or more times. Straight growth was measured by one of two methods; 1. direct measurement of the difference between the initial and final total length; or 2. measurement of the increase in length of a zone extending from the tip to a charcoal-petroleum jelly mark placed 6 mm from the tip following chemical treatment. The second method was preferred since the measured growing region then coincided with the zone responding to the geotropic stimulus. The first method was employed only for the shoots, since the elongation zone extends over a considerable length. In the case of corn shoots two growth centers are involved; the coleoptile tip, and the rib meristem below the apical meristem.

Results

Interaction of NP and IAA

Over the range of 2.86 to 11.4×10^{-7} moles of IAA per liter and NP concentrations of 10^{-5}, 10^{-4}, and 5×10^{-4} M, there was no competitive interaction between IAA and NP (Figure 1) in Avena section growth. At high IAA concentrations (5.72 to 115×10^{-6} M) and 5×10^{-4} M NP, competitive inhibition was observed (Figure 2). Lower concentrations of NP were not tested competitively owing to their ineffectiveness in inhibiting the geotropic response. The inhibition of pea root section growth caused by 2.86 to $28.6 \times$
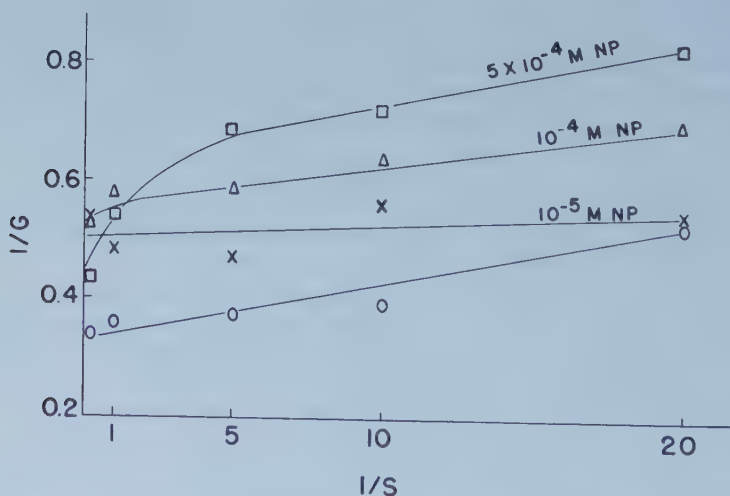
Figure 1. *Interaction in Avena coleoptile section growth test of IAA and NP.* $1/S=1/IAA$ concentration in mg/l. $1/G=1/$growth in mm/section/12 hr
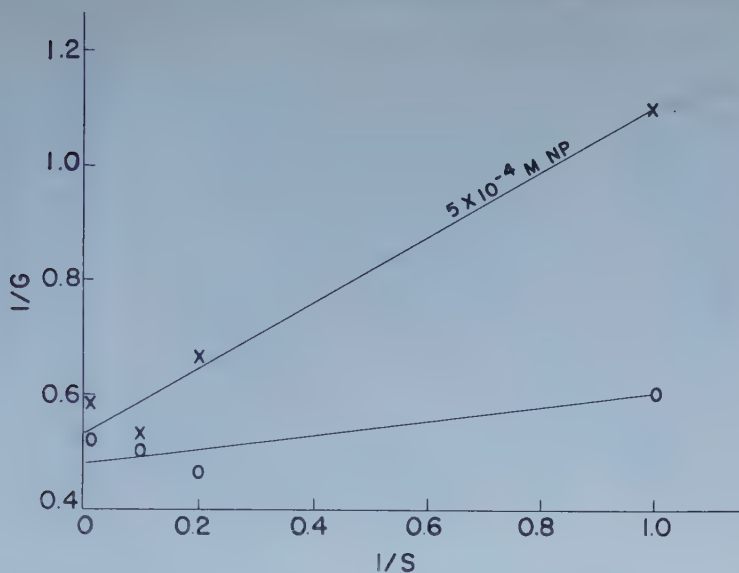
Figure 2. *Interaction of IAA and NP in Avena coleoptile section growth test at high IAA concentrations.* 1/S=1/IAA concentration in mg/l. 1/G=1/growth in mm/section/12 hr.

10^{-7} M IAA was not relieved by 5×10^{-4} M NP. Epinastic effects have been observed in tomato plants treated with 10^{-7} M NP (Hoffmann and Smith). Thus NP cannot unambiguously be classified as a growth promoting substance or as a competitive inhibitor of IAA action.

It should be pointed out that the competitive inhibition data may not hold true for shorter time intervals. A striking example of this is the 2.6 substituted phenoxy acetic acids (Osborne *et al*) which were active in extension growth when measurements were made after short time intervals.

Time course of straight growth and geotropic curvature

The time course of straight growth and geotropic curvature in control materials was studied in order to select a minimal incubation period on the linear portion of the time-response curve and of easily measurable magnitude. Results for the several plant materials used are summarized in Figures 3—7. In all the plant materials tested, growth is a linear function of time for at least four hours. The geotropic curvature reaches a maximum at 4 hours (earlier in corn roots), then remains constant or sometimes decreases as the bending zone becomes mature. These results indicate that under the conditions employed, a direct correlation between rate of straight growth and rate of geotropic curvature exists.
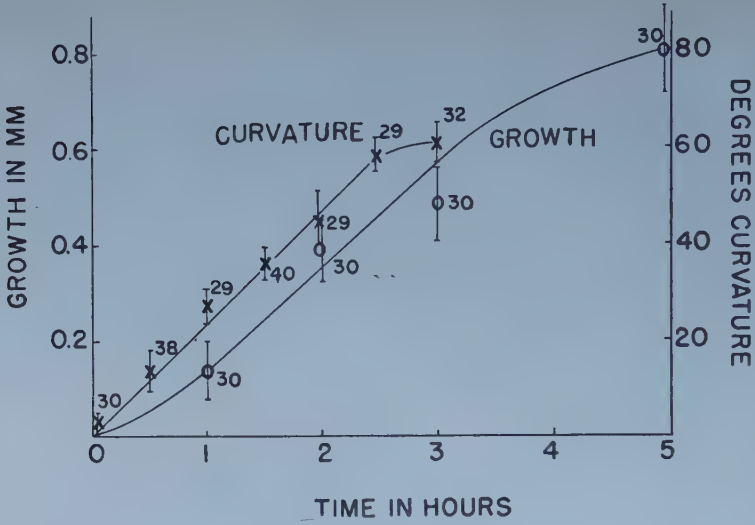
Figure 3. *Time course of growth and geotropic curvature of Avena coleoptiles.* Vertical lines crossing each point represents ± 2 X the standard error. The number of plants used is shown for each point.
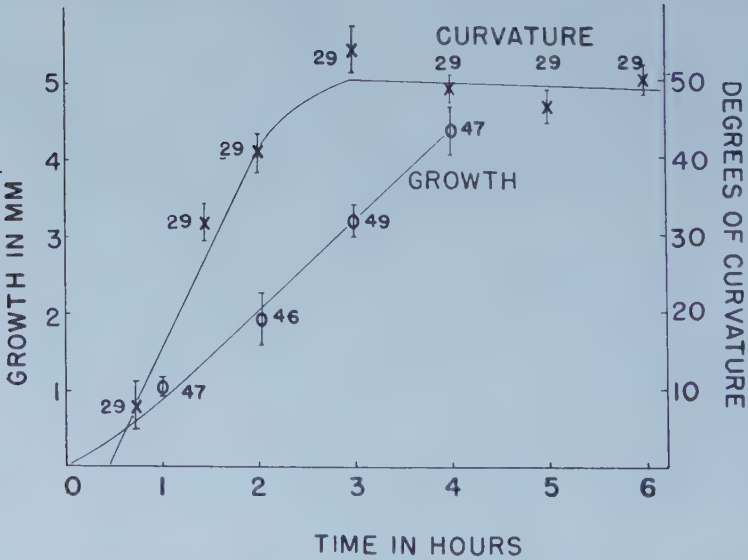
Figure 4. *Time course of growth and geotropic curvature of corn roots.*
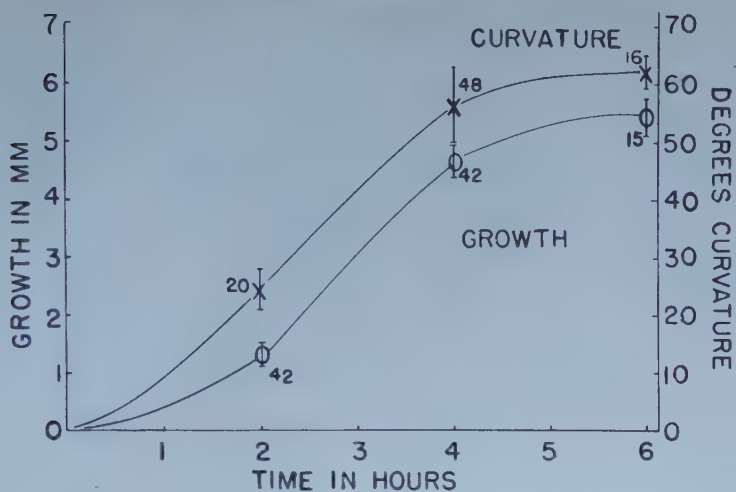
Figure 5. *Time course of growth and geotropic curvature of corn shoots.*
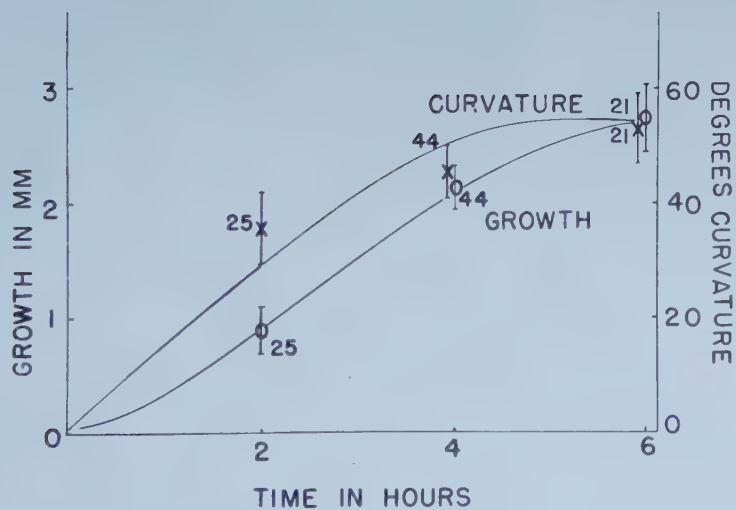
Figure 6. *Time course of growth and geotropic curvature of pea roots.*
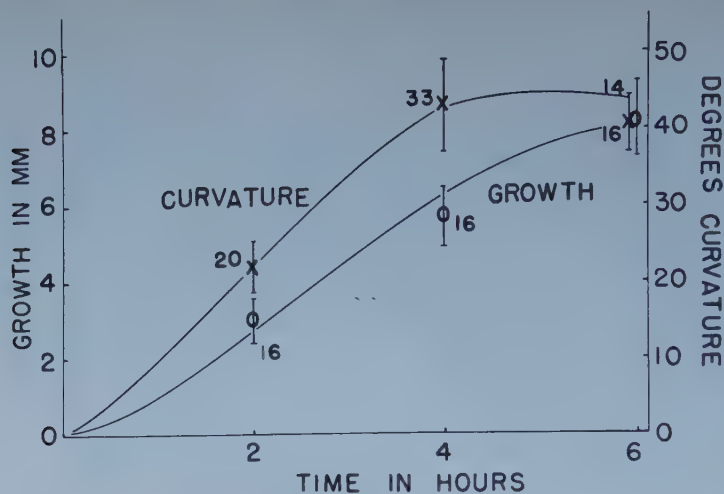
Figure 7. *Time course of growth and geotropic curvature of pea shoots.*

Disproportional inhibition of straight growth and geotropic curvature of shoots and roots of corn and pea

Straight growth and geotropic curvature of shoots and roots of corn and pea were determined after a 4 hour incubation following a one hour pre-treatment of the plant material with the various concentrations of NP. The experimental data for each plant material tested are calculated as percent of the control and plotted in Figures 8—11. Low concentrations of NP, e.g. 10^{-5} *M*, inhibit both geotropic curvatures and straight growth of shoots
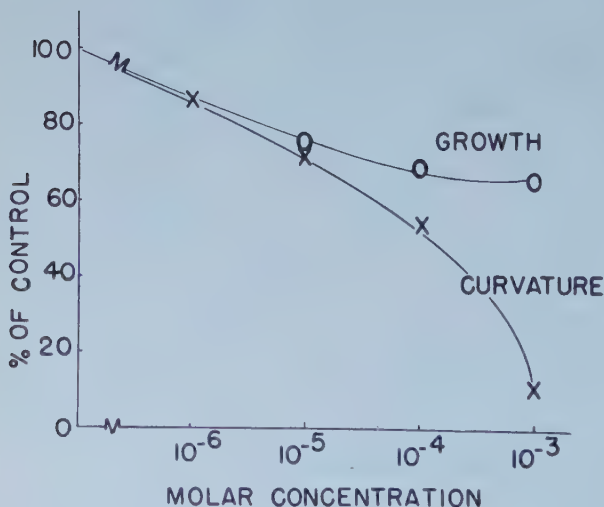
Figure 8. *Straight growth and geotropic curvature of NP pre-treated corn shoots expressed as percent of the buffer treated control.*
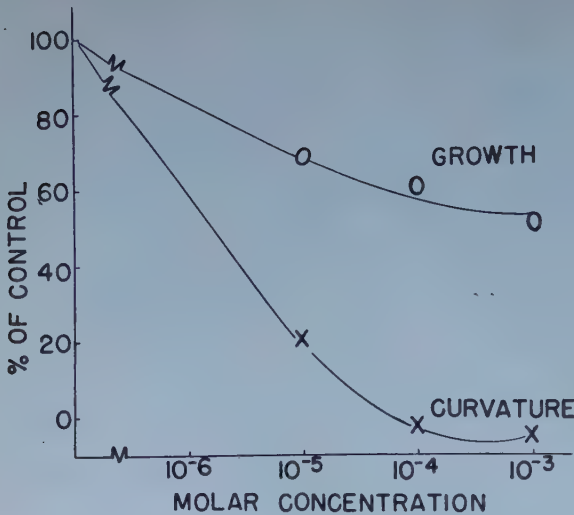
Figure 9. *Straight growth and geotropic curvature of NP pre-treated corn roots expressed as per cent of the buffer treated control.*
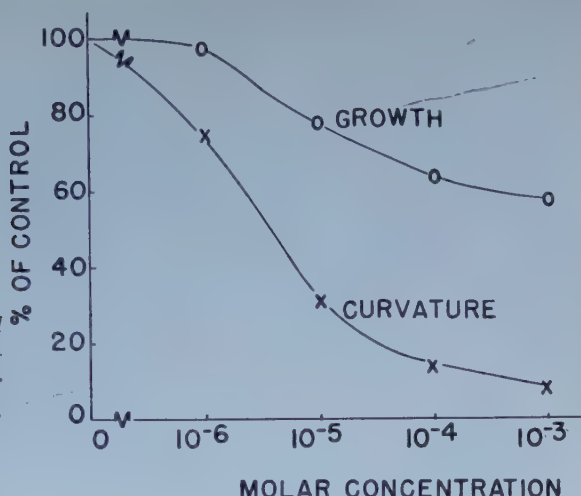
(Figures 8 and 10) by approximately 30 percent. At 10^{-4} and 10^{-3} M a marked disproportionality is manifested; 10^{-3} M causing an almost total inhibition of the geotropic curvature of shoots and only a 40 percent inhibition of straight growth. A somewhat similar situation characterizes the curvature and straight growth of root tissue (Figures 9 and 11) except that the disproportionality becomes evident at lower inhibitor concentrations. A 70 to 80 percent inhibition of the tropic response is caused by 10^{-5} M NP, whereas straight growth is inhibited only 20 to 20 percent.
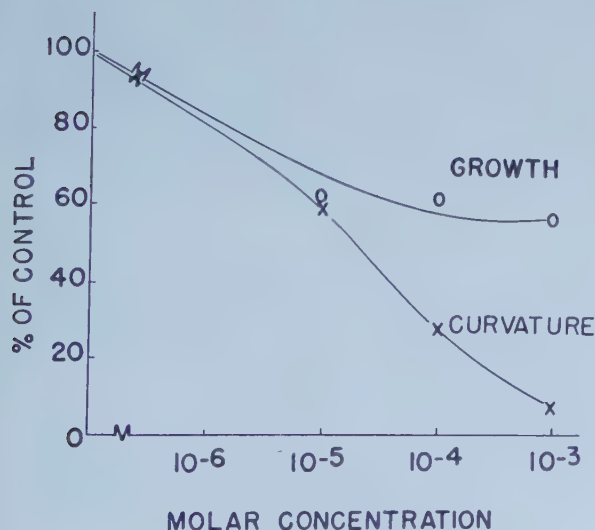
Figure 10. *Straight growth and geotropic curvature of NP pre-treated pea shoots expressed as per cent of the buffer treated control.*

Figure 11. *Straight growth and geo-tropic curvature of NP pre-treated pea roots expressed as per cent of the buffer treated control.*

The concentrations inducing 50 per cent inhibition of each response for each plant organ, as estimated graphically, are summarized in Table 1. This peculiar, "uncoupling" of growth and geotropic curvature by NP occurs in root and shoot tissues of both monocotyledonous and dicotyledonous plants.
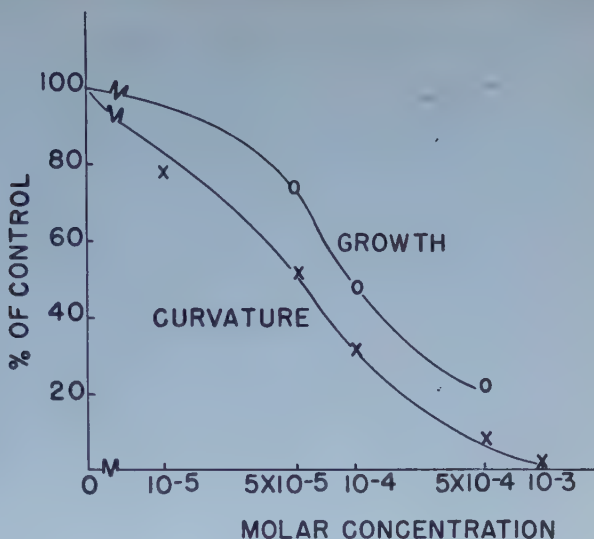
Proportional inhibition of straight growth and geotropic curvature of the Avena coleoptile by NP

Since Avena coleoptiles are very sensitive to growth regulators, and their physiological behavior is well known, earlier experiments were performed using intact Avena coleoptiles. It was found that NP inhibition was proportional to the concentration of the chemical used, and for a single concentration, inhibition increases with increasing time of incubation. Data for a 90 minute incubation following a 30 minute pretreatment are plotted in Figure 12.

Table 1. Concentrations in *M* of NP and DCIBA required for one-half inhibition of straight growth and geotropic curvature in different plant organs.

Material	Chemical	Growth	Curvature
Corn roots	DCIBA	1×10^{-4}	1×10^{-4}
Avena coleoptile	NP	1×10^{-4}	5×10^{-5}
Corn roots	NP	$> 1 \times 10^{-3}$	4×10^{-6}
Corn shoots	NP	$> 1 \times 10^{-3}$	1×10^{-4}
Pea roots	NP	1×10^{-3}	$< 1 \times 10^{-5}$
Pea shoots	NP	$> 1 \times 10^{-3}$	2×10^{-5}

Figure 12. *Straight growth and geotropic curvature of NP pre-treated Avena coleoptiles expressed as per cent of the buffer treated control.*

There is a direct parallelism between inhibition of straight growth and inhibition of geotropic curvature in this tissue. Avena coleoptile tissue thus responds differently from the other plant materials tested.
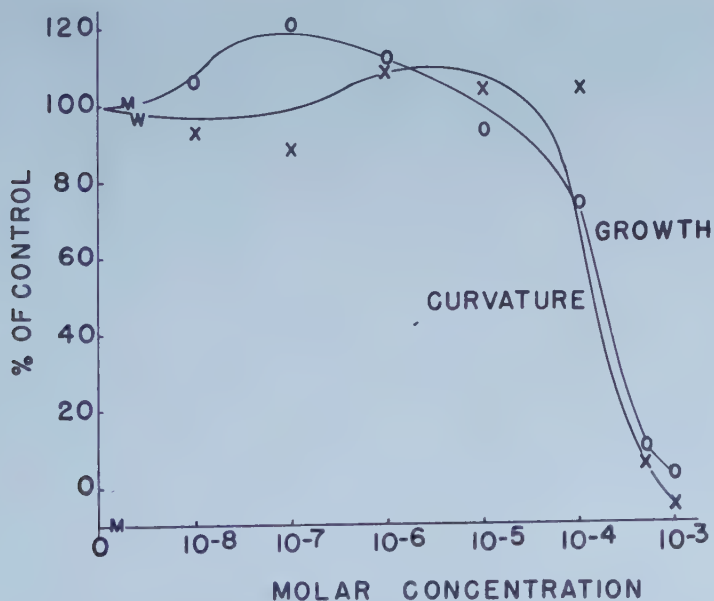

Figure 13. *Straight growth and geotropic curvature of DCIBA pre-treated corn roots expressed as per cent of the buffer treated control.*

Proportional inhibition of growth and geotropic curvature of
corn roots by DCIBA and IAA

For comparison with the results of NP-treatment, 2,4-dichlorophenoxyiso-butyric acid (DCIBA) was tested for inhibition of both growth and geotropic curvature. DCIBA has been reported to stimulate wheat root growth (Burström, 1951) and to competitively inhibit IAA action in the Avena section test (McRae and Bonner 1953). DCIBA, thus, has some of the properties associated with antiauxins. The experimental data are plotted as percent of the control in Figure 13. A direct correlation of straight growth and geotropic curvature is clearly obtained. Proportional inhibition apparently is not a general property of antiauxins, since Brumfield (1955) observed a disproportional inhibition of growth and geotropic curvature in timothy roots following treatment with another antiauxin, 2,4,6-trichlorophenoxyacetic acid.

The effects of IAA on growth and geotropic curvature of corn roots were examined, and in confirmation of results recently presented by Brumfield, IAA treatment results in proportional inhibition of growth and geotropic curvature.

Discussion

The geotropic response of plant organs may be visualized as occuring in three steps: *1.* the perception of the stimulus; *2.* the transport of the stimulus or a product resulting from that stimulus; and *3.* the growth response. Either the elongation zone or the tip may serve as the perception zone as shown by the experiments of Keeble, Nelson and Snow (1929) and more recently by Anker (1954). Loss of geotropic sensitivity following decapitation is due, apparently, to removal of the source of growth substance. There is no general agreement as to the mechanism of the gravitational perception or the transport of stimulus subsequent to the perception. It will suffice for the present purposes to consider the theory proposed by Went and Cholodny. These authors believe that gravity affects a displacement of growth substances, and that the resultant asymmetric distribution causes differential growth of the upper and lower sides of the plant organ; thus bending occurs. A direct correlation between growth and geotropic curvature may thus be expected and, as a first approximation, inhibition of straight growth should always result in proportional inhibition of curvature. This expectation is experimentally realized in the case of DCIBA and IAA inhibition of root growth and curvature and is also apparent from the correlation between growth and geotropic curvature in untreated control material.

The selective inhibition of geotropic curvature by NP is difficult to

explain. It is further difficult to understand the dissimilarities in behaviour of Avena coleoptile tissue, which shows proportional inhibition of straight growth and curvature, and the roots and shoots of corn and peas, which show disproportional inhibition. Our present data are insufficient to determine the mechanism of the selective tropic inhibition by NP. Three possibilities present themselves assuming the validity of the Went-Cholodny theory: 1. An effect of NP upon the lateral transport of endogenous auxin; 2. An effect of NP upon the perception of the gravitational stimulus; or 3. An effect of NP upon only that fraction of growth associated with curvature and assuming two components contributing to growth.

We believe that experiments recently reported by Anker cast some doubt upon the importance of a lateral redistribution of auxin as a mechanism for the geotropic bending. Anker found that the geotropic response of decapitated Avena coleoptiles could be restored by incubation of the decapitated coleoptile sections in solutions containing IAA, indoleacetonitrile and naphthyl acetic acid. He did not interpret his results as being at variance with the Went-Cholodny theory, and further, did not determine internal auxin concentrations. It would appear that redistribution of auxin by coleoptiles immersed in a homogenous solution of IAA is improbable, and therefore an effect of NP upon internal auxin redistribution would not be a likely explanation.

An effect of NP upon the perception mechanism is similarly open to objection. NP has been reported by Nétien and Conillot to inhibit the phototropic response. These authors did not present quantitative data to show that the inhibition was not due simply to an inhibition of growth by NP. In view of the results reported here, however, it would seem that NP may inhibit growth and the phototropic response disproportionally. Since the perception of the photostimulus and the perception of the geo-stimulus probably involves dissimilar mechanisms, it seems unlikely that NP acts to inhibit both sensory mechanisms.

The possibility was entertained that straight growth consists of two components, and only one of these growth components is effective in producing curvature. If then NP were to inhibit only that fraction of growth normally associated with curvature, a disproportional inhibition of straight growth and curvature would be observed. This explanation appears unlikely because NP at 10^{-6} M significantly inhibits curvature with no inhibition of straight growth (Figure 9).

We are therefore left without a suitable explanation of the mode of NP action. Our current concept is simply that NP prevents the local acceleration and/or inhibition of growth caused by gravity. The elucidation of the mechanism of this action remains for future studies.

Summary

1. Over the time interval for geotropic bending to reach a maximum, a direct correlation exists between the rate of straight growth and geotropic curvature in untreated Avena coleoptiles and the young roots and shoots of corn and pea.

2. A disproportional inhibition of growth and curvature was found in *n*-1-naphthylphthalamic acid (NP) treated roots and shoots of corn and pea, but no disproportionality was observed in NP treated Avena coleoptiles.

3. Corn roots treated with 2,4-dichlorophenoxyisobutyric acid showed a proportional inhibition of straight growth and geotropic curvature.

4. The close correlation between straight growth and geotropic curvature to be expected on the basis of the Went-Cholodny theory was not obtained.

References

Anker, L.: A comparative study on the recovery of the geotropic response of decapitated Avena coleoptiles by indoleacetic acid, indoleacetonitride and naphthylacetic acid. — K. Nederl. Akad. Wetenshap. Proc. Sec. C. 57: 304. 1954.

Brumfield, R. T.: The inhibition of curvatures in timothy roots by certain chemicals. — Am. J. Bot. 42: 958. 1955.

Burström, H.: Studies on growth and metabolism of roots. VI. The relative growth action of different isobutyric acid derivatives. — Physiol. Plant. 4: 470. 1951.

Grigsby, B. H., Tsou, Te May, & Wilson, G. B.: Some effects of Alanap (NP) and amizole on germinating peas. — Proc. 11th North Central Weed Control Conf. 88. 1954.

Hoffmann, O. L. & Smith, A. E.: A new group of plant growth regulators. — Science 109: 588. 1949.

Keeble, F., Nelson, M. G. & Snow, R.: The intergration of plant behavior. I. Separate geotropic stimulations of tip and stump in roots. — Proc. Roy. Soc. London, Sec. B. 105: 493. 1929.

McRae, D. H., Foster, R. J. & Bonner, J.: Kinetics of auxin interaction. — Plant Physiol. 28: 343. 1953.

McRae, D. H., & Bonner, J.: Chemical structure and antiauxin activity. — Physiol. Plant. 6: 485. 1953.

Mentzer, C., Molho, D., & Pacheco, H.: Relations entre la structure chemique et l'inhibition des tropismes chez les vegetaux. — Bull. Soc. Chim. Biol. 32: 572. 1950.

Mentzer, C., & Nétien, G.: Sur un procede permettant de troubler le geotropisme des racines. — Bull. Mens. Soc. Linneenne. Lyon, 19: 102. 1950.

Nétien, G., & Conillot, R.: Action de lacide naphthyl-phtalamique sur la germination et les Premiers stades de croissance des végétaux. — Bull. Mens. Soc. Linneenne, Lyon, 20: 49. 1951.

Osborne, Daphne, J., Blackman, G. E., Nova, S., Sudzuki, F., & Powell, R. G.: The physiological activity of 2,6-substituted phenoxy acetic acids. — Jour. Exp. Bot. 6: 392. 1955.

Went, F. W., & Thimann, K. V.: Phytohormones. — MacMillan Co., N. Y. 1937.

Absorption Spectra of the Anthocyanin Pigment of Red Cabbage: a Natural Wide-range pH Indicator

By

FREDERICK T. WOLF

Department of Biology, Vanderbilt University, Nashville, Tenn.
(Received May 26, 1956)

Introduction

Fuld (1905) discovered that the pigment of red cabbage has the properties of a pH indicator. He described this finding as follows: "A happy observation has given me an indicator with an intermediate acid and alkali sensitivity — beautiful red in acid solutions, green in alkaline — which can be prepared directly from red or blue cabbage." Walbum (1913 a, 1913 b) reported similar observations made independently. In a later publication, however, he acknowledged the priority of Fuld's discovery (Walbum, 1913 c).

The color changes which the pigment undergoes with change in pH have been described qualitatively by a number of workers, including Walbum (1913 b), Milobedzki and Jajte (1926), Brauner (1929), Willstaedt (1931), Popescu (1934 a), and Grube, Diekmann, and Gundermann (1943). The observations of these investigators, which are in substantial agreement, indicate that solutions of the anthocyanin pigment from red cabbage are red at pH 1.0—3.0, violet or purple at pH 5.0—6.0, blue at pH 7.0, green at pH 8.0—9.0, and yellow at pH 10.0—11.0. The pigment of red cabbage is therefore unusual among anthocyanin compounds in displaying such a diversity of color states. In general, the anthocyanin pigments are water-soluble pH indicators which change from red to purple to blue, but do not become green or yellow. It has therefore been suggested that the red cabbage pigment has advantages over other anthocyanins for use as a pH indicator.

Fuld (1905) found that the colors of red cabbage anthocyanin solutions were thermostable. Walbum (1913 b) found that proteins, neutral salts,

toluene and chloroform did not cause interference, and that this indicator gave satisfactory agreement with electrometric determinations. A characteristic which lessens the possibilities for use of this indicator in biological systems is the fact that the anthocyanin is destroyed by polyphenol oxidase (Jorgensen and Vejlby, 1953), an enzyme of rather wide occurrence in biological materials.

The pigment is rather easily extracted from cabbage leaves by a variety of methods. Popescu (1934 a) used hot water for extraction. Ethanol has been the reagent of choice of most workers, including Walbum (1913 b), Milobedzki and Jajte (1926), and Grube, Diekmann and Gundermann (1943). In the chemical work of Chmielewska (1933) and Frey-Wyssling and Blank (1943), dried cabbage was extracted with methanol containing 2 per cent HCl. A solution of KCl in 1/100 N HCl was also used by the latter investigators.

Perhaps the most satisfactory method of isolation of the anthocyanin was that employed by Frey-Wyssling and Blank (1943). Red cabbage leaves, dried and ground, were first extracted for 16 hours with methanol containing 2 per cent HCl. Following filtration, the cabbage meal was again extracted with methanol-HCl, and the extracts were pooled. The pigment was precipitated from the methanol solution by the addition of two volumes of ether; the precipitate was collected and was dissolved in warm methanol. After standing for one day, a precipitate of inorganic salts separated spontaneously, and was discarded.

The pigment was reprecipitated from methanol by the addition of 4 volumes of ether, and was then dissolved in water. Addition of neutral lead acetate to the aqueous solution of the pigment until it became blue-violet resulted in precipitation of the lead salt of the anthocyanin. After standing for 24 hours, this precipitate was recovered by filtration, and was washed with methanol to remove inorganic impurities. The lead salt of the anthocyanin was then decomposed by the addtion of 3 per cent HCl, and the lead chloride which precipitated was removed by filtration. Final precipitation was accomplished by the addition of 10 volumes of ether. A yield of 8—9 gm of anthocyanin per kilo was obtained by this method.

In spite of a considerable amount of chemical work, the structure of the red cabbage anthocyanin is not yet completely elucidated. Chmielewska (1933) determined the empirical formula of the anthocyanidin as $C_{28}H_{33}O_{16}Cl$. Upon hydrolysis with 20 per cent HCl, it was shown that the red cabbage anthocyanidin was a derivative of cyanidin, and glucose was identified as a substituent sugar group. Upon extraction of an ether solution with Na_2CO_3, sinapic acid (4-hydroxy, 3,5-dimethoxycinnamic acid) was identified. Willstaedt (1935) confirmed the empirical formula proposed by Chmielewska, and showed that the molecule contains one or more methoxyl groups.
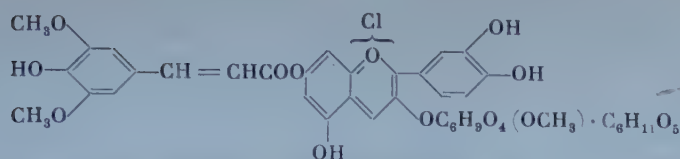
Figure 1. *The structural formula of the anthocyanin pigment of red cabbage* (after Chmielewska, Smardzewska, and Kulesza, 1938, as modified by Frey-Wyssling and Blank, 1943).

Chmielewska (1936) designated the red cabbage pigment as rubrobrassyl chloride or rubrobrassicin chloride. Following degradation, the methyl ester of sinapic acid was isolated, and it was established that the pigment molecule contains one mole of sinapic acid. Chmielewska, Smardzewska and Kulesza (1938) concluded that the structure of rubrobrassyl chloride was that of a methyl ether of 5- (or 7-) sinapoyl cyanidin chloride bioside. Of the two sugar residues, one is glucose, while the other is a different unidentified sugar. This concept of the structure of the red cabbage anthocyanin is shown in Figure 1.

It has been suspected that the wide variety of colors exhibited by red cabbage extracts might be due to a mixture of substances. Blue and yellow colors of alkaline solutions were ascribed by Brauner (1929) to accompanying tannins or flavones. Frey-Wyssling and Blank (1943) chromatographed the red cabbage pigment on a column of alumina, gypsum and talc, and from its behavior found it to be homogenous. The blue and yellow colors of alkaline solutions must therefore be ascribed to the anthocyanin itself. Upon hydrolysis of red cabbage extracts with 20 per cent HCl. Frey-Wyssling and Blank (1943) report that two anthocyanidins are produced, which differ in solubility in hot dilute HCl and in methanol.

The absorption spectra of rubrobrassyl chloride have been previously determined by Brauner (1929) and Popescu (1934 a, 1934 b). For the red pigment at pH 3, Brauner (1929) reported an absorption maximum of 500—515 mμ. At pH 6, the absorption band for the violet phase becomes broader and is shifted toward the red, maximum absorption occurring at 530—616 mμ. At pH 8, the absorption of the blue pigment is further shifted, to the region of 555—645 mμ. Popescu (1934 a determined the absorption spectra of 1 per cent aqueous solutions by means of a spectrograph and a microphotometric procedure. pH was not carefully controlled in Popescu's studies and the results are reported in terms of percentage absorption The absorption maximum of an acid solution was found at 520 mμ Maxima for a neutral solution were reported at 514, 554, and 577 mμ, and for an alkaline solution at 500 mμ. In a later paper, Popescu (1934 b) presented ultraviolet

absorption spectra, as well as fluorescence spectra. Reinhold (1937) employed colorimetry in the quantitative determination of the red cabbage anthocyanin.

The present paper is concerned with the absorption spectra of the red cabbage anthocyanin in the visible region. Measurements were made under carefully controlled conditions of pH. The findings are regarded as an essential prerequisite to the use of rubrobrassyl chloride as a pH indicator.

Materials and Methods

Heads of red cabbage, *Brassica oleracea* L. var. *capitata* L. f. *rubra* L., were obtained at a local market. These were cut into small pieces, and the stems, which contain little pigment, were discarded. Preliminary trials at preparation of the pigment were made by the method of Chmielewska (1933) and Frey-Wyssling and Blank (1943), which involves extraction in methanol containing 2 per cent HCl and precipitation from the methanol solution by the addition of 2 volumes of ether. The resulting precipitate proved to be very gummy and hygroscopic, for which reason this procedure was not considered further.

Extraction was therefore performed in boiling water. The colored extract was filtered, and solid lead acetate was added to the hot solution until no further precipitate resulted. The addition of lead acetate to the red solution resulted in a color change to blue-violet. After standing in the cold for 24 hours, the precipitate of the lead salt of the anthocyanin was collected by centrifuging and decantation. The precipitate was washed with methanol, and was then dissolved in a minimal amount of distilled water. Five per cent sulphuric acid was then added to decompose the lead salt. The addition of sulphuric acid was continued until no further precipitate of lead sulphate was produced. The lead sulphate was then removed by centrifugation. The clear red solution of the anthocyanin was stored in a refrigerator, and aliquots were adjusted to various pH values by the addition of concentrated NaOH or HCl.

pH measurements were made with a Beckman Model G pH meter. Absorption measurements were made with a Beckman Model DU spectrophotometer, using distilled water as a blank, and are reported in terms of optical density.

Results

The findings are presented in Figures 2—6 and in Table 1. At pH 1.0, there is a very pronounced absorption peak at 530 mμ. At pH 2.0, there is no shift in the position of the peak, but its height has declined. At pH 3.0, the height of the absorption peak has further declined, and the position of the absorption maximum begins to shift slightly toward the red. At pH 4.0, the shift toward the red had become pronounced, the absorption maximum now being at 545 mμ.

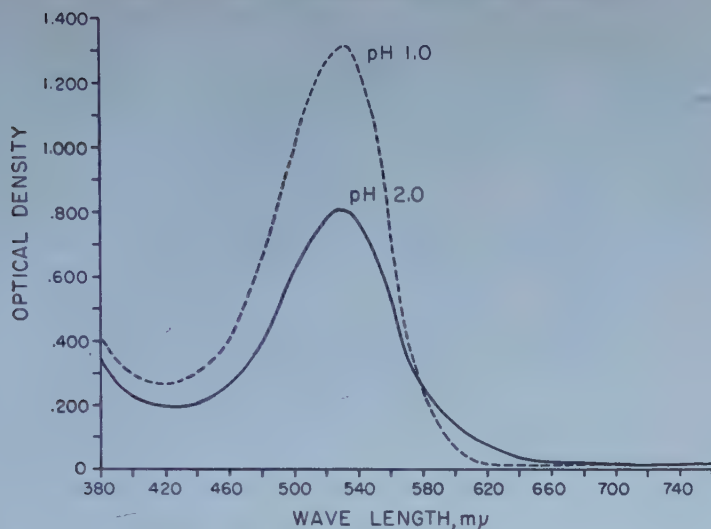With further increases in pH, the absorption peak becomes further and

Figure 2. *The absorption spectrum of red cabbage anthocyanin at pH 1.0 and 2.0.*
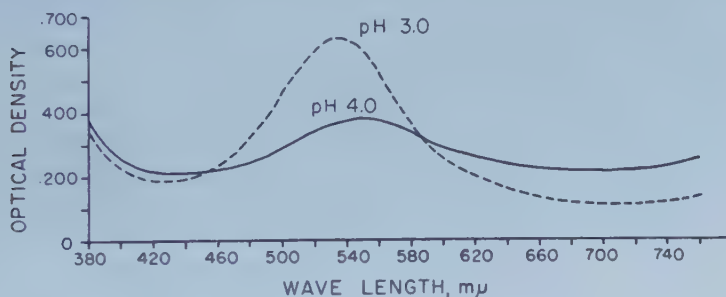

Figure 3. *The absorption spectrum of red cabbage anthocyanin at pH 3.0 and 4.0.*
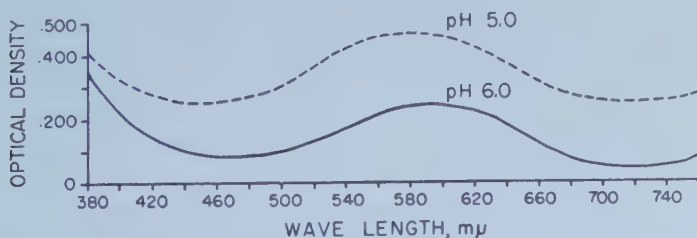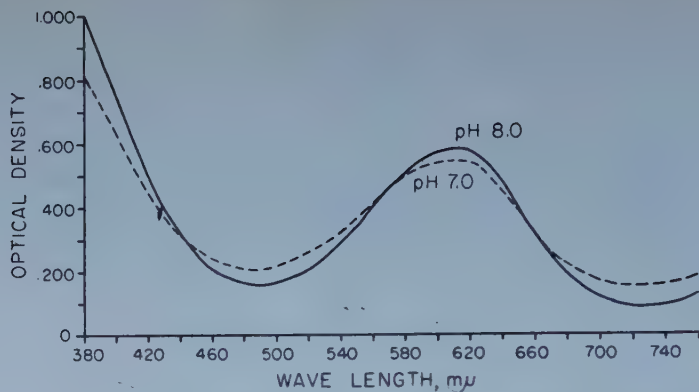

Figure 4. *The absorption spectrum of red cabbage anthocyanin at pH 5.0 and 6.0.*

further shifted toward the red end of the spectrum, until at pH 9.0, the peak is located at 615 mμ. At pH 10.0, accompanying the color change of the anthocyanin from green to yellow, this absorption peak has been obliterated, and maximal absorption now occurs at much shorter wave lengths, below 420 mμ.

37

Figure 5. *The absorption spectrum of red cabbage anthocyanin at pH 7.0 and 8.0.*
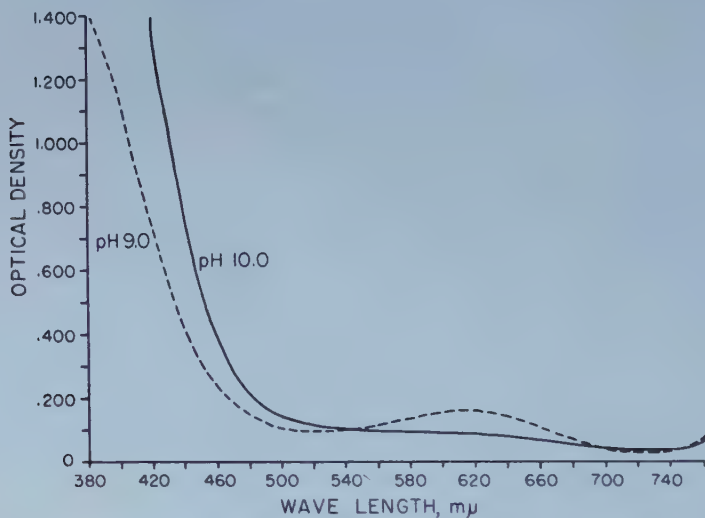
Figure 6. *The absorption spectrum of red cabbage anthocyanin at pH 9.0 and 10.0.*

Discussion

The present findings concerning the absorption spectrum of the red cabbage anthocyanin are in good agreement with the qualitative data of Brauner (1929), but they give no indication of three maxima for the neutral solution at 514, 554, and 577 mμ, as reported by Popescu (1934 a, 1934 b). Similarly, the position of the absorption maxima found at various pH values on the alkaline side differs greatly from the value of 500 mμ which Popescu observed.

Table 1. *The colors and absorption maxima of the anthocyanin pigment of red cabbage as a function of pH.*

pH	Color of solution	Absorption maximum, mμ
1.0	Red	530
2.0	»	530
3.0	Purplish red	535
4.0	Reddish purple	545
5.0	Purple	580
6.0	Blue	590
7.0	»	605
8.0	Bluish green	610
9.0	Green	615
10.0	Yellow	< 420

In general, anthocyanin pigments usually vary in color with pH from red in acid solution to purple at neutrality to blue in alkaline solution. The red color is generally considered to be due to the oxonium salt of the pigment, while the purple and blue colors are related to quinone formation in the ring not included in the benzopyrylium nucleus. The purple phase is usually considered as representing the color base, and the blue phase as the salt of the color base (Link, 1943; Blank, 1947; Bonner, 1950). In the red cabbage anthocyanin, the situation is clearly more complex, through the existence of the green and yellow color phases. The questions as to which of the five color phases correspond to the oxonium salt, to the color base, and to the salt of the color base in the usual anthocyanins, and the related question concerning the structures to be assigned to the phases which do not so correspond, must remain unanswered at this time.

Summary

This study is concerned with the absorption spectra of the anthocyanin pigment of red cabbage, *Brassica oleracea* L. var. *capitata* L. f. *rubra* L. in the visible region. The pigment was obtained in a state of relative purity by a procedure consisting of extraction in boiling water, precipitation of the anthocyanidin as the lead salt, and decomposition of the lead salt with acid. Absorption spectra of aqueous solutions were determined at pH values ranging from 1.0 to 10.0.

With change in pH, the pigment varies in color from red to purple to blue to green to yellow. At pH 1.0 and 2.0, there is an absorption peak at 530 mμ. As the pH is increased, the maximal absorption becomes shifted to longer and longer wave lengths, maximum absorption at pH 9.0 being at 615 mμ. At still higher pH values, this peak becomes obliterated, and maximal absorp-

-tion in the visible then occurs below 420 mµ. These spectral properties indicate that the red cabbage anthocyanin has great potential usefulness as a naturally occurring pH indicator which covers a very wide range.

References

Blank, F.: The anthocyanin pigments of plants. — Botan. Rev. 13: 241. 1947.

Bonner, J.: Plant Biochemistry. — xvii plus 537 pp., Academic Press, New York. 1950.

Brauner, L.: Das kleine pflanzenphysiologische Praktikum. I Teil. — 112 pp. G. Fischer, Jena. 1929.

Chmielewska, Irena: Badania nad barwnikiem czerwonej kapusty (*Brassica oleracea*). 1. Etude sur le colorant du chou rouge. I. — Roczniki Chemji 13: 725. 1933.

—   Badania nad barwnikiem czerwonej kapusty (*Brassica oleracea*). II. Etude sur le colorant du chou rouge. II. — Roczniki Chemji 16: 384. 1936.

—   I. Smardzewska & J. Kulesza: Badania nad barwnikiem czerwonej kapusty (*Brassica oleracea*). III. Studien über den Rotkohlfarbstoff. III. — Roczniki Chemji 18: 176. 1938.

Frey-Wyssling, A. & F. Blank: Untersuchungen über die Physiologie des Anthocyans in Keimlingen von *Brassica oleracea* L. var. *capitata* L. f. *rubra* (L.). — Ber. Schweiz. Botan. Gesell. 53 A: 550. 1943.

Fuld, E.: Ueber einen neuen Indikator. — Muenchener Med. Wochenschr. 52: 1197. 1905.

Grube, O., K. Diekmann & R. Gundermann: Der Farbstoff des Rotkohls als Indikator in pH Bereich 8,5—10. — Chem. Zeitung 67: 34. 1943.

Jorgensen, E. & K. Vejlby: A new polyphenol oxidase test. — Physiol. Plant. 6: 533. 1953.

Link, K. P.: The anthocyanins and the flavones. — In Gilman, H. Organic Chemistry, an advanced treatise, Vol. II, Ch. XVIII, pp. 1315—1340, John Wiley, New York. 1943.

Milobedzki, T. & S. Jajte: O stosowaniu wyciagu z modrej kapusty jako wskaznika. (Use of extracts of blue cabbage as an indicator). — Roczniki Chemji 6: 177. 1926.

Popescu, I. G.: Absorption dans le spectre visible des solutions de tournesol (*Rocella Tinctoria*) et des colorants du chou rouge. — Bul. Soc. Romône de Fizica 36: 1. 1934 a.

—   Spectres d'absorption et de fluorescence des colorants du tournesol et du chou-rouge (Deuxieme note preliminaire). — Bul. Soc. Romône de Fizica 36: 83. 1934 b.

Reinhold, J.: Kolorimetrische Bestimmung des Anthozyangehaltes bei Rothkohl. — Landw. Jahrb. 84: 833. 1937.

Walbum, L. E.: Sur l'emploi de l'extrait de choux rouge comme indicateur dans la mesure colorimétrique de la concentration des ions hydrogène. — Compt. Rend. Trav. Lab. Carlsberg 10: 227. 1913 a.

—   Über die Verwendung von Rotkohlsauszug als Indicator bei der colorimetrischen Messung der Wasserstoffionenkonzentration. — Biochem. Zeitschr. 48: 291. 1913 b.

—   Nachtrag zu meiner Arbeit: Über die Verwendung von Rotkohlsauszug als Indicator bei der colorimetrischen Messung der Wasserstoffionenkonzentration. — Biochem. Zeitschr. 50: 346. 1913 c.

Willstaedt, H.: Über den Farbstoff des Rotkohls. I (vorläufige) Mitteilung. — Biochem. Zeitschr. 242: 303. 1931.

—   Über den Farbstoff des Rotkohls. II. — Biochem. Zeitschr. 276: 262. 1935.

Some Observations on the Growth Substances in Ether Extracts of the Potato Tuber

By

W. G. Burton

Ditton Laboratory, Department of Scientific and Industrial Research
(Received June 8, 1956)

Introduction

Appleman (1918) suggested that the sprouting of potatoes might be controlled by their content of growth substances, although little direct evidence for this suggestion was obtained until many years later. Guthrie (1939) investigated the possibility that dormancy might be due to the inhibition of bud growth by a high content of auxin, and concluded that the amount of auxin present in dormant tubers was too small for this to be the case. Michener (1942) suggested that auxin inhibited bud growth in the dormant tuber, but this conclusion was reached from experiments on the effect of treatment with ethylene chlorhydrin upon the auxin content. Michener stated that he had done no experiments to determine the conditions bringing about the end of the rest period in untreated tubers. The first intensive work upon the naturally occurring growth substances of the potato tuber was that of Hemberg. The conclusions which he finally reached from his results (Hemberg 1942—1954) were briefly as follows: —

In ether extracts of potato peel there were two growth promoting and two growth inhibiting substances — an acid and a neutral substance in each case. Dormancy resulted from the presence of the acid growth inhibiting substance and this disappeared rapidly after harvest. The neutral growth inhibiting substance was still present in non-dormant tubers. The concentration of acid growth promoting substance was low, and was still low immediately after the end of the dormant period. In Spring it began to increase and reached

a maximum just before bud elongation was noticeable. The concentration of the neutral growth promoting substance in the peel of unsprouted potatoes was also low but was much greater in the peel of sprouted potatoes.

The methods employed by Hemberg, in that they distinguish between acid and neutral substances, may be open to criticism on the following grounds. The neutral inhibiting substance was estimated by its inhibition of the promotion of indolyl acetic acid of Avena coleoptile curvature, while the acid inhibiting substance, presumed to be masked by the acid promoting substance, was estimated from "activity curves" in which the weight of peel represented in the extract was plotted against coleoptile curvature. These activity curves (see more particularly Hemberg, 1952) provided evidence that both promoting and inhibiting substances were present in the acid fraction, but their form could be explained by postulating a constant content of inhibiting substance and an increasing content of promoting substance, rather than a constant content of promoting substance and a decreasing content of inhibiting substance (compare, for example, the curves published by Larsen, 1947). Hemberg (1951) has stated that, from other evidence, this alternative explanation may be discounted in the case of his potato extracts, but nevertheless an estimation based on other methods would seem desirable. The concentration at which inhibition by the acid substance could be demonstrated was also very high. Thus, the neutral inhibiting substance, supposed to have no influence upon the dormancy of the potato, showed an appreciable inhibiting effect at a concentration of equivalent to about 1 g. of peel per ml. agar; while the "activity curves" of the acid fraction (Hemberg, 1952) showed no effect necessarily attributable to the presence of an inhibiting substance until a concentration of some 20 g. peel per ml. agar was reached. The division into "acid" and "neutral" fractions may also be criticized because the method employed (Boysen-Jensen 1941) does not necessarily produce such a separation and is open to other objections (see p. 571).

The use of ether for the extraction of growth substances is widespread. It possesses the advantage that it is readily removable by evaporation; it has the great disadvantage (if it is the only solvent used in an investigation) that a number of substances of physiological importance, such as 7-hydroxy-6-glucoxy-coumarin, ascorbic acid and chlorogenic acid, which are present in plants, are insoluble in it, or practically so. The methods used by Hemberg further restricted the substances he extracted and tested to those which were soluble in ether, chloroform and water (in which substances diffuse in agar). In addition to these objections to the solvents used, the Avena test itself may be of doubtful significance in assessing substances which are of importance in the break of dormancy of the potato — a process in which cell division is initially of primary importance. Coumarin for example, is a

substance which will inhibit the growth of the adventitious roots on potato sprouts grown *in vitro* in concentrations at which its effect on Avena coleoptile elongation is comparatively slight.

Lengthy extraction by ether will tend to be accompanied by the enzymatic formation of auxins, but at the temperatures at which our extractions, and those of Hemberg, were done such auxin formation might be expected to be slight (see Wildmann and Muir, 1949). On the other hand enzymatic reactions are clearly proceeding during the extraction, as shown by the formation of melanin, the amount formed appearing to vary with the variety and storage history of the tubers. This may be regarded as a serious objection to the techniques employed.

Despite these objections the fact remained that the disappearance of an ether soluble growth inhibiting substance had been observed to be correlated with the break of dormancy, and it seemed to be a matter of some importance to substantiate this.

Methods

The method of extraction adopted was very similar to that employed by Hemberg [1] though it differed in detail. The tubers were washed and peeled into ether contained in a bolt-head flask which was then placed in the dark at $1°$ C. Replicate extractions were not made. In most of the work reported here the peel from about $1 \frac{1}{2}$ kg. of potatoes was extracted, though in some experiments 7—8 kg. were used. On three successive days after this the ether was decanted into a dark bottle kept at $1°$ C. and replaced by fresh ether. On the fourth day the flask (still containing the last addition of ether) and the bottle were transferred to $-20°$ C. for about 24 hours. As a result of this much of the water dissolved in the ether was deposited as ice and the inclusion of water-soluble but ether-insoluble substances in the extract was avoided to a large extent. The ether was decanted from the ice, and from the frozen peel and sap in the flask, into a distilling flask and evaporated off under reduced pressure. The oily residue was taken up in the minimal amount of chloroform, by four washings of the flask, and kept in a dark bottle at $-20°$ C. Whenever possible the separation and assay of the extract was started immediately, as in some previous work there had been evidence of change in extracts stored in transparent bottles at $-20°$ C.

Although in the earliest work at the Ditton Laboratory on the growth substances of the potato the Avena curvature test was used, in all the work reported here the straight growth method has been employed. This was as follows: —

The grains (var. 172) were soaked, without removal of the husks, for 24 hours in tap water in a covered Petri dish at $15°$ C. and then planted in silver sand (80 per cent of which lay in size between 100 and 150 μ) brought to a moisture

---

[1] I had the opportunity of working with Dr. Hemberg for a short time in 1947. I should like to take this opportunity of putting on record my thanks to Prof. Stålfelt and Dr. Hemberg for the hospitality extended to me.

·content of about 15 per cent by the addition of tap water. The sand was contained in $2'' \times 1''$ specimen tubes held in stands which were covered by crystallizing dishes (19 cm. diam.; 10 cm. deep) the walls of which were lined with wet filter paper. The tubes were placed at $25°$ C. for about 66 hours, the first 24 hours being in the dark and thereafter under phototropically inactive red light. The seedlings were then about 2.5 cm. high. The terminal 3 mm. of the coleoptiles was cut off, as for the curvature test, leaving the leaf undamaged, and the seedlings left a further 6 hours by which time the leaf had grown sufficiently to be grasped easily with the thumb and forefinger. Each seedling was harvested at this stage, the terminal 8 mm. of the coleoptile cut off, the leaf withdrawn from it, and the coleoptile cylinder placed in distilled water in a Petri dish. Cylinders were withdrawn from the bulked sample, measured, and placed in the test solutions contained in 6.5 cm. covered Petri dishes. Each Petri dish was subdivided by 16 mm. diam. glass rings, the individually measured coleoptiles thus being kept separate. Measurement was done by placing the cylinders 21 at a time on a sub-divided slide and projecting their images ($\times 3.5$) onto photographic paper. The shadowgraphs were developed and the images measured to the nearest millimetre with a flexible steel rule. After 24 hours in the test solutions the cylinders were re-measured. The controls were placed in a solution containing 1 per cent sucrose and $1/100$ M KH_2PO_4; the test solutions consisted of the same solution plus growth substances eluted from a chromatogram.

As mentioned above, the validity of the Avena test may be questioned as a means of assaying substances which are concerned in the break of dormancy of potatoes. For this reason some work has been done upon the possibility of using potato sprouts grown *in vitro* as test material. The method used was derived from that developed by Mes and Menge (1954) for growing stem segments *in vitro*. It did not prove to be as useful a test as had been hoped, for reasons given below, but some relevant results were obtained from it and it is therefore described.

Sturdy sprouts about 3—4 cm. in length — sprouts of the varieties Home Guard and Craig's Defiance were found to be most suitable — were broken from the parent tubers, weighed and measured, and submerged in a 0.1 per cent solution of mercuric chloride for exactly 5 minutes. (It is essential that the sample of sprouts used in any one experiment should be uniform. A supply of sprouts may be maintained by storing seed tubers at $5°$ C. and putting them to sprout at $10°$ C., as required, in an atmosphere with a water V.P.D. of about 2 mm. of Hg. (c. 80 per cent R.H.). Under these conditions adventitious roots are not present initially.) The sprouts were then transferred to sterile water in a covered sterile container, and from this another covered bath of sterile water. From this they were taken to be planted in sterile agar in $6'' \times 1''$ test tubes which were closed with cotton wool plugs. All normal precautions were taken in handling the sprouts — the forceps used were sterilized by alcohol and flaming, the mouths of the test tubes were flamed before and after planting, which was done by dropping the sprouts base end downwards into the agar, and all operations were done in a small draught-free room. The agar medium consisted of 0.7 per cent agar, 1 per cent sucrose and $1/100$ M KH_2PO_4 made up with distilled water or with water elutates from chromatograms. Some media were made using potato sap instead of water and omitting the sucrose and KH_2PO_4. 10 ml. of medium were placed in each test tube, and the tubes were then plugged and autoclaved at 1 atm. pressure for 20—30 minutes. The tubes with the planted sprouts were placed in a bin containing moist cotton

wool at 25° C. in the dark. The sprouts increased in length and weight, and there was a vigorous production of adventitious roots, the number of these produced by each sprout of a uniform sample being reasonably constant. Branching did not occur in the case of Home Guard and Craig's Defiance sprouts, but could be induced by very high concentrations of β-indolyl acetic acid. Unfortunately the increase in length and weight during the comparatively short duration of the experiments — 6 or 7 days — did not show any likelihood of being of use as a means of assay. Measurements of root growth seemed to show some promise of being of use, and some examples are given in Table 1 illustrating the effect of coumarin, ascorbic acid and supra-optimal concentrations of β-indolyl acetic acid. The total length of roots was perhaps the best index, in that it reflected changes in both number and length. The only advantage possessed by this test over the conventional root-growth tests is that it employs as test material the species of plant which is under investigation. I feel this advantage to be of great importance because of the markedly different response of different plants to growth substances. The response of *Parthenium argentatum* and *Lycopersicum esculentum* to *trans*-cinnamic acid may be quoted as an example of this. The potato root-growth test as used by us possesses three disadvantages however. First is the possibility of chemical change in growth substances during autoclaving. Because of this, when comparing this test with the Avena straight growth method, the culture solutions used in the latter were autoclaved at the same time as the culture media used in the former. Secondly, the roots are not produced under uniform conditions, some originating above the surface of the agar and some below it. Thus, for example, the roots which apparently grew in high concentrations of coumarin as shown in Table 1 were all produced above the surface of the agar and ceased growth on reaching that surface. The third disadvantage lies in the different responses of roots and shoots to any given concentration of an active substance. This is common to the use of any root growth test in a study of shoot growth. It seems reasonable, however, to assume that lack of inhibition of root growth may indicate the absence of substances which would inhibit shoot growth.

It is not altogether certain to what extent a plant extract should be subdivided before assay. In much of the published work extracts have been divided into so-called 'acid' and 'neutral' fractions and the latter has frequently been discarded. The methods of separation into 'acid' and neutral fractions have usually been based on those developed by Boysen-Jensen (1941) and are open to several objections. In the first place, sub-division is by no means always into acid and neutral fractions. Acids may pass wholly into the acid fraction, as does for example *trans*-cinnamic acid; but neutral substances will be divided between the acid and neutral fractions according to their coefficients of partition between ether and an alkaline saturated solution of glucose. An example of this is 7-hydroxy-6-methoxycoumarin, which occurs in amounts of the same order in both the acid and neutral fractions. Recently another fault of the commonly used method of subdivision has been demonstrated by Larsen (1955) who has shown that tartaric acid, which has been used for re-acidification and release of the acid growth substances, may give rise to a substance which inhibits the growth of Avena coleoptiles. An objection to the use of any method of division lies in the fact that if we divide an extract we necessarily test the growth substances, as it were, out of context; and the more effective the means of separation, the more this will be so. This objection applies, of course, with equal force to the use of selective solvents, as has been mentioned in the

Table 1. *Growth of adventitious roots on potato sprouts grown on agar at 25° C.*

Growth promoter or inhibitor added to the agar	No. of adventitious roots on 5 sprouts	Total length of roots (cm.)	Average root length (cm.)	Total length of roots (Control ≡100)
1. Control for 2—4	94	164.2	1.75	100
2. 11.8 mg./l. coumarin	75	101.4	1.35	62
3. 29.6 ,, ,, ...	61	51.7	0.85	31
4. 59.2 ,, ,, ...	46	35.3	0.77	22
5. Control for 6—7	154	240.6	1.56	100
6. 100.5 mg./l. coumarin	28	17.5	0.63	7
7. 201 ,, ,, ...	20	9.1	0.46	4
8. Control for 9—14	84	107.3	1.28	100
9. 10 mg./l. ascorbic acid	81	111.6	1.38	104
10. 20 ,, ,, ,, ...	92	99.0	1.08	92
11. 60 ,, ,, ,, ...	98	118.5	1.21	111
12. 125 ,, ,, ,, ...	110	135.9	1.23	127
13. 250 ,, ,, ,, ...	141	139.5	0.99	130
14. 500 ,, ,, ,, ...	54	57.4	1.06	53
15. Control for 16—21	117	153.3	1.31	100
16. 0.05 mg./l. β-indolyl acetic acid	92	113.4	1.23	74
17. 0.1 ,, ,, ,, ,,	80	102.7	1.28	67
18. 0.2 ,, ,, ,, ,,	74	87.1	1.18	57
19. 0.5 ,, ,, ,, ,,	69	89.2	1.29	58
20. 25 ,, ,, ,, ,,	70	79.0	1.13	52
21. 100 ,, ,, ,, ,,	36	42.7	1.19	28

Varieties of sprout used: — 1—7, Craig's Defiance grown for 7 days; 8—14 Dr. McIntosh grown for 6 days; 15—21, Craig's Defiance grown for 6 days. Although Dr. McIntosh sprouts were used in one of these experiments, they are not really suitable, being comparatively weak and bent. The two experiments on the effect of coumarin used samples of sprouts of different size; hence the large difference in the production of roots by the controls — the average weight of sprout in No. 1 was 0.832 g., and in No. 5 was 1.432 g.

introduction. Interactions of growth substances have been studied by several workers (see e.g. Skoog *et al.,* 1942) and a substance which springs to mind in relation to studies on the potato is ascorbic acid. This was found by Bennet-Clark (reported by Ball, 1953) to reduce the stimulating effect of β-indolyl acetic acid upon Avena coleoptile elongation, an effect which had also been observed by us. Dehydroascorbic acid may, on the other hand, have a stimulating effect (Raadts and Söding, 1947). In fact freshly harvested potato tubers may well contain sufficient ascorbic acid (c. 500 mg./l. sap) to cause marked inhibition of coleoptile elongation even in the presence of 0.5 mg./l. β-indolyl acetic acid (Figure 1), but this ascorbic acid will be absent from an ether extract.

The present paper is concerned only with an investigation of a correlation reported to exist between the break of dormancy and the disappearance of an ether-soluble inhibiting substance, and for this reason interactions between water-soluble and ether-soluble extractives will not be discussed further. For the same reason it was thought justifiable to fractionate the ether extracts in a way such that the inhibiting effect was clearly detectable. All the routine separations have been done on filter

Figure 1. *Influence of ascorbic acid upon the stimulating effect of β-indolyl acetic acid.* Concentration of β-indolyl acetic acid 0.5 mg./l.
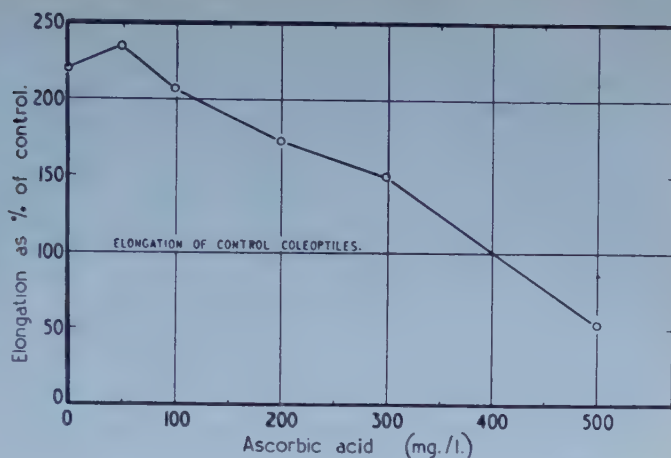
paper by partition or adsorption chromatography, although, using potatoes of the variety Stormont Dawn in 1952, we found very good separation of an inhibiting substance on a 'Dowex II' resin column.

Much of our early work on chromatographic separation was devoted to choosing suitable solvents and determining the R_F's of physiologically active substances in the potato. If any solvent remains on the paper (or, conceivably, reacts with the paper) it may itself have a marked effect upon coleoptile elongation — for example if a chromatogram is run in 10 per cent acetic acid and then dried in a current of air at 110° C. for 2 hours, the elutate from the whole chromatogram will inhibit elongation. On the other hand, drying which is sufficiently prolonged completely to remove the solvent may affect the growth substances. For this reason it was decided that the most suitable method of separating growth substances might be by adsorption on filter paper using water as the solvent. It is not necessary in this case to dry the paper, provided a correction is made for dilution by water retained by it.

Glass chromatography tanks are used, approximately 30 cm. × 20 cm. × 55 cm. high, the rim being ground to provide good contact with the plate glass top. Strips of filter paper supported at the top and dipping into water — or, in the case of mixed solvents, into the aqueous phase — in the bottom of the tank maintain a uniformly saturated atmosphere. The solvent is contained in a stainless steel trough supported about 5 cm. below the top of the tank. The filter paper used is Whatman No. 3, as this thick paper will hold a comparatively large amount of extract. As cut for use, the sheets are rather more than 50 cm. long and any suitable width from about 5 to 20 cm. A starting line is drawn 10 cm. from one end of the paper and the extract (in chloroform solution) streaked slowly along it from a 1 ml. graduated pipette, with a current of air blowing over the paper in order that the band produced does not spread to a width greater than 1 cm. The paper is then placed in the tank in the dark at 20° C., with no solvent in the trough, to equilibrate overnight. The next day distilled water is poured into the trough through a small hole provided in the plate glass top, this hole being closed with a bung during equilibration and running. The paper is removed and cut up when the solvent front has advanced about 35 cm. — which takes about 3 1/2–4 hours. Normally in testing a fresh extract for activity the chromatogram is cut transversely into six
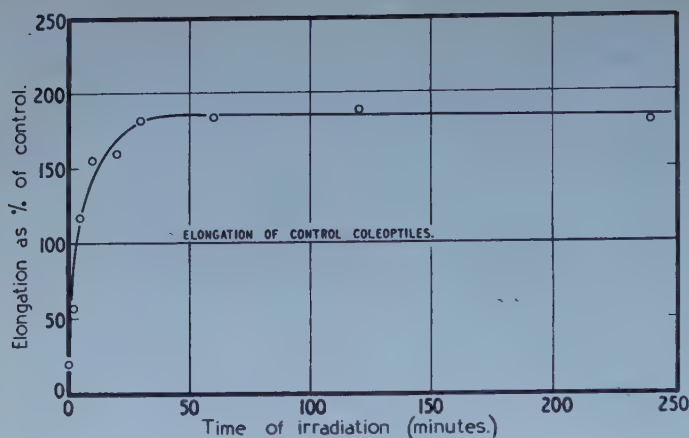
Figure 2. *Effect of irradiation of trans-cinnamic acid with UV light (2537 Å, 30 watt)* upon its influence upon Avena coleoptile elongation. *Concentration of cinnamic acid, 25 mg./l. Exposed in solution 4 mm deep, the top surface being 3 cm. from the lamp filter.*

equal pieces, sufficient of the paper before the starting line being taken to include the spread of the starting band. If activity is established in any zone, further subdivision of the relevant part of another chromatogram may give a more accurate estimate of the R_F of the substance involved, though because of the width of the starting band and a certain amount of 'tailing' of the substances present it is pointless to reduce the width of the fractions to less than about 3 cm. Paper from before the starting line or after the solvent front has sometimes been used as a control but such controls have not been found to differ from pure culture solution. Nor have blank papers, run in water and eluted, shown any zones of physiological activity in our experiments.

Using the above procedure it is normal for the chromatograms to have a perfectly straight solvent front and for the R_F's of identifiable substances to be reasonably consistent. For example, there is always present a substance, provisionally identified as scopoletin, which exhibits an intense light blue fluorescence under ultraviolet light. The R_F of this substance in a series of 14 chromatograms varied between 0.26 and 0.30. It may be mentioned here that at the beginning of this work it was customary to view the chromatograms under ultra-violet light before cutting them up. This practice was discontinued because of the comparative rapidity with which ultra-violet light will cause molecular re-arrangement in some physiologically active substances. As an example of this, the change of *trans*-cinnamic acid to *cis*-cinnamic acid is illustrated in Figure 2 (see also Zimmerman and Hitchcock, 1939, with reference to the activation of *trans*-cinnamic acid, tryptophane and β-naphthoylacetonitrile).

Each strip of the chromatogram was cut into small pieces and placed with 15 ml. of culture solution in a stoppered 25 ml. conical flask and shaken in the dark at 25° C. for about 24 hours. The solutions were then decanted into Petri dishes for test as described above.

Chromatograms prepared from ether extracts of potato run in water as above, show two zones of physiological activity — a diffuse 'promoting' zone between R_F 0.35 and 0.6, and an 'inhibiting' zone between R_F 0 83 and 1.0. It is of course not claimed that these zones represent single substances, but they represent two clearly defined regions of activity which respond consistently to changes in storage

conditions, and which have been found to be present in extracts from all the varieties — ten in number — which have been studied. In the work reported here an attempt has been made to determine whether a relationship exists between the start of sprouting and the changes observed in the activity exhibited by the two zones. For the purposes of routine work the 'promoting zone' was taken as being from R_F 0.3 to R_F 0.65, and the "inhibiting zone" from R_F 0.65—R_F 1.0. It should be mentioned that this "inhibiting zone" includes the inhibiting substance present in so-called "acid fractions" as separated by the method of Boysen-Jensen (1941).

Results

Examples of the affects upon coleoptile extension of elutates of the promoting and inhibiting zones are given in Figure 3. All the extracts have been assessed at three or more concentrations as shown. The comparisons below are based on the effects of concentrations equivalent to 3 g. of peel per ml. of culture.

Changes in the inhibiting and promoting substances from different varieties during storage. — Comparisons were made of the inhibiting zones derived from extracts of four varieties during storage at 10° C. They represented the extremes in sprouting behaviour as found among commercial varieties and were kindly provided by Mrs. N. McDermott from crops grown from Scotch seed at the Nottingham University School of Agriculture, Sutton Bonington. The sprouting behaviour of the varieties is given in Table 2, and Figures 4 and 5 show the changes in, respectively, the inhibiting and promoting zones derived from them at different times during storage.

The effect of storage temperature upon the inhibiting and promoting substances. — Samples of the variety Dr. McIntosh grown from Scotch seed at the East Malling Research Station were placed at a number of storage temperatures the day after they were harvested. Their sprouting behaviour is

Table 2. *Sprouting behaviour of potatoes stored 1954—55.* Weeks in store.

Variety and storage temp. °C.	Sprouts g./tuber							
	0.0 but eyes 'open'	0.1	0.5	1.0	2.0	4.0	6.0	8.0
Arran Consul, 10°	11	23	27	30	33	—	—	—
Craig's Defiance, 10°	5	6	8	9	12	17	21	—
Golden Wonder, 10°	11	16	18	20	22	—	—	—
Home Guard, 10°	5	7	11	16	18	20	22	—
Dr. McIntosh, 15°	9	11	14	15	18	21	23	26
Dr. McIntosh, 25°	3	5	6	7	9	12	16	22

Dr. McIntosh put into store, 6.10.54. Other varieties, 5.10.54. In the case of Dr. McIntosh stored at 5° C. the eyes were 'open', i.e. the first signs of sprout growth were observed, after 14 weeks' storage, but the sprouts did not grow sufficiently to be weighable.
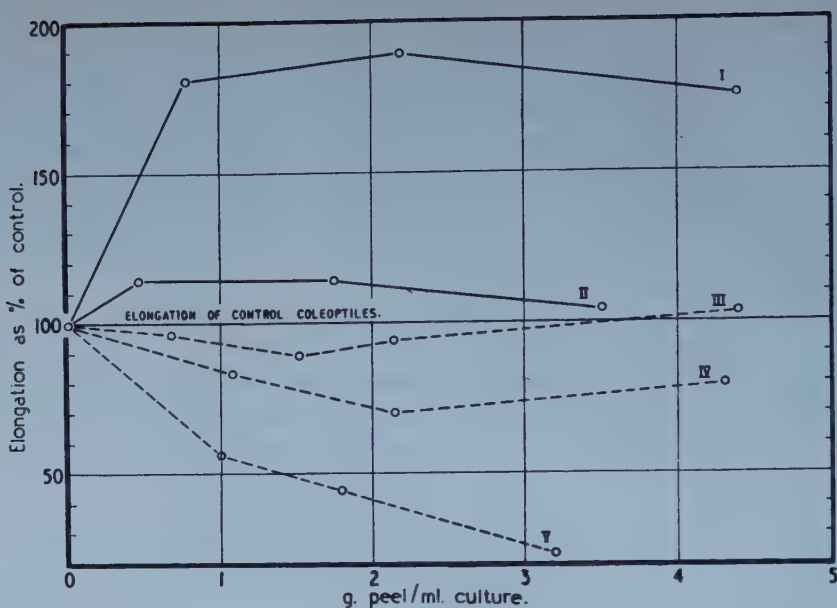
Figure 3. *Effect upon Avena coleoptile elongation of elutates of the promoting and inhibiting zones of paper chromatograms prepared as described in the text.* Variety of potato: — Majestic stored at 5° C. Harvested 30.10.53.
I. Promoting zone 12.5.54. II. Promoting zone 11.1.54. III. Inhibiting zone 12.5.54. IV. Inhibiting zone 10.2.54. V. Inhibiting zone 2.11.53.

shown in Table 2 and the changes in the inhibiting and promoting zones in Figures 6 a and b respectively.

Comparison between the effects upon Avena coleoptiles and upon potato sprouts in vitro. — For the purposes of this comparison the elutates of the inhibiting zones were present in the agar, in which the sprouts were grown, at the same concentration in terms of g. peel/ml. as in the culture solution in which the coleoptiles were grown. Elutates from the promoting zones were not compared because preliminary trials with autoclaved cultures had indicated that there might be some destruction of the activity of this zone during sterilization of the agar. There was no evidence, in the few trials done in the course of this work, that the activity of the inhibiting zone was similarly impaired. As an example of the results obtained, in one experiment tests of inhibition using extracts of two samples of the variety Golden Wonder at a concentration of 1.25 g. peel/ml. culture or agar gave the following results, expressed as percentages of the control values: —

Extract made after storage for 3 weeks at 10° C.: coleoptile elongation, 54; number of roots, 98; total length of roots, 94; average root length, 95.
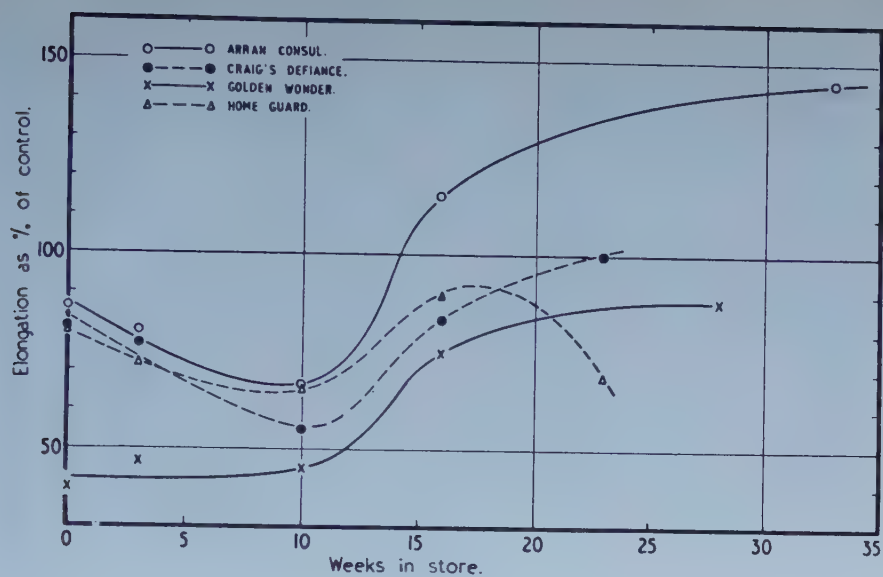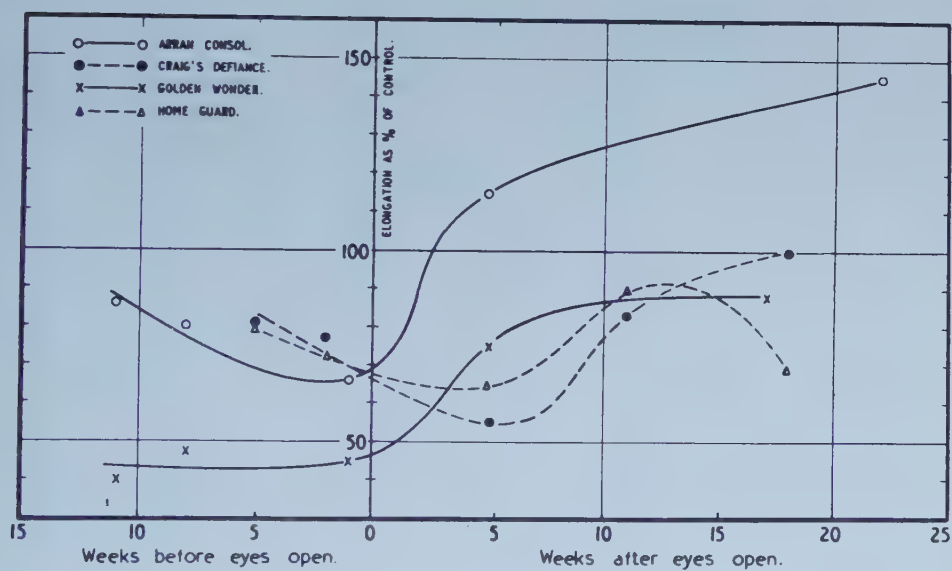
Figure 4 A.

Figure 4 B.

Figure 4. *Effect upon Avena coleoptile elongation of elutates of the inhibiting zone of paper chromatograms of extracts prepared from tubers stored for different periods at 10° C. Concentration of elutates equivalent to 3 g. peel/ml. culture.*
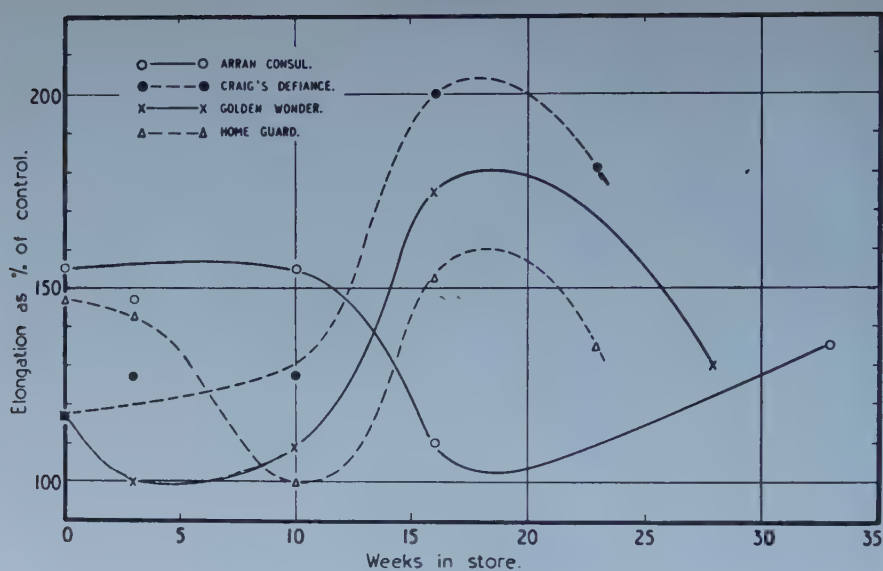A. Related to total time of storage.
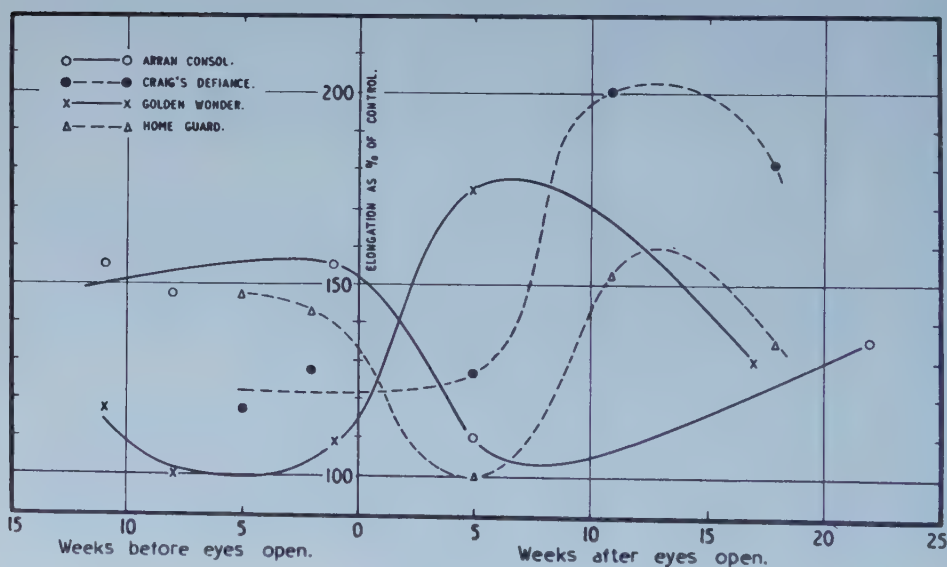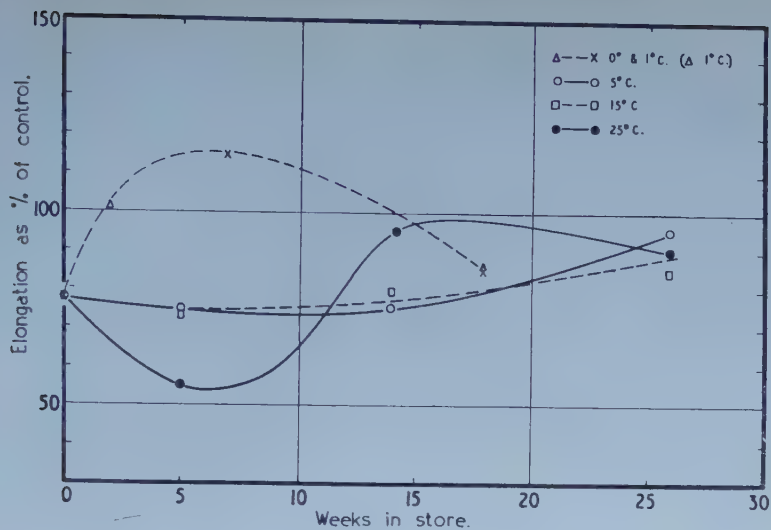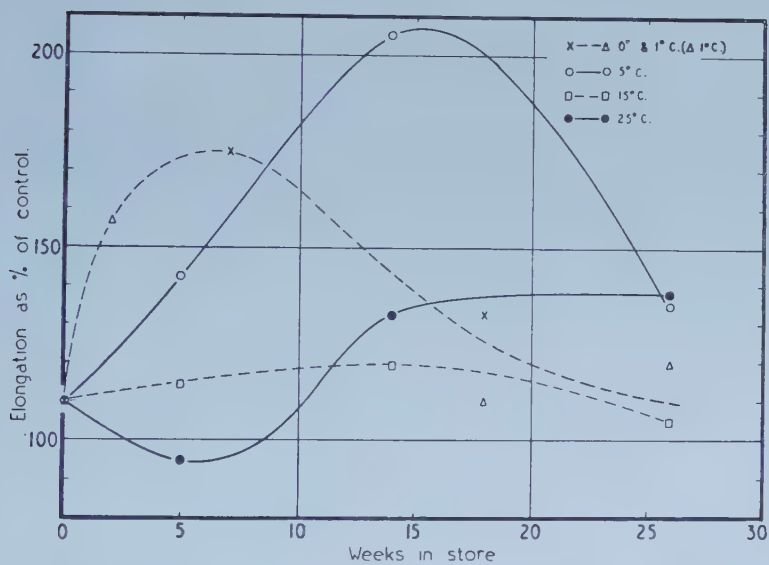B. Related to the start of sprouting.

Figure 5 A.

Figure 5 B.

Figure 5. *Effect upon Avena coleoptile elongation of elutates of the promoting zone of paper chromatograms of extracts prepared from tubers stored for different periods at 10° C.* Concentration of elutates equivalent to 3 g./peel/ml. culture.

A. Related to total time of storage.

B. Related to the start of sprouting.

Figure 6 A.

Figure 6 B.

Figure 6. *Effect upon Avena coleoptile elongation of elutates of paper chromatograms of extracts prepared from tubers stored at various temperatures.* Variety of potato: Dr. McIntosh. Concentration of elutates equivalent to 3 g peel/ml. culture.

A. Inhibiting zone.

B. Promoting zone.

Extract made after storage for 16 weeks at $10°$ C.: coleoptile elongation, 82; number of roots, 101; total length of roots, 110; average length, 109.

Discussion

It is clear from the above results that ether extracts of potato peel contain substances which inhibit Avena coleoptile elongation, and substances which promote it. These substances fluctuate in amount during storage, and the nature of the fluctuation is conditioned by storage temperature. It seems very probable that the two parts of the adsorption chromatogram which show activity may each contain a mixture of growth promoting and growth inhibiting substances. The form of the successive dilution curves (Figure 2) during storage would support this view as would the replacement of inhibition by quite marked promotion in the 'inhibiting' zone of Arran Consul after prolonged storage (Figure 4). Changes in the activity of the zones may be due to changes in either promoting or inhibiting constituents — for instance loss of inhibition may result from an increase in a promoting substance. It would be quite possible for the inhibition to decrease while the concentration of actual inhibiting substances was increasing. Bearing this in mind let us consider the behaviour of each zone separately.

Inhibiting zone. If we first consider the varieties stored at $10°$ C. (Figure 4) we find a tendency for inhibition to increase, except in the case of the variety Golden Wonder in which it was considerable from the beginning of storage. After 10 weeks' storage, however, this trend was reversed and there was a marked loss of inhibition. It should be noted that there was no sign that loss of inhibition was a necessary pre-requisite for sprouting to occur — for instance, in the variety Craig's Defiance the maximum inhibitory effect was found when the tubers already had sprouted to the extent of between 1 and 2 grams per tuber. It is true that in both Arran Consul and Golden Wonder the loss of inhibition (and in the case of the former, the appearance of promotion) followed hard upon the first observable signs of growth, suggesting that perhaps a promoting substance appeared at this time, but this was not the case in the varieties Craig's Defiance and Home Guard. It is in fact much easier to relate this loss of inhibition to time of storage than to sprouting. As mentioned above the loss first started to occur after 10 week's storage in every case, this corresponding to 15 weeks after the haulm had been burnt off. This similarity of behaviour in relation to time of storage closely resembles the strikingly similar behaviour of the 20 varieties of potato studied by Allison and Driver (1953) with respect to the loss of ascorbic acid. With four exceptions (the varieties Arran Pilot, Arran

Chief, Inverness Favourite and Glen Ilam) the content of ascorbic acid had decreased to a minimum 15 weeks after harvest. In three of the exceptions the content of ascorbic acid after 15 weeks differed only slightly from the minimal value recorded. In the other the minimal value had already been reached 10—11 weeks after harvest. It is tempting to suggest that the loss of inhibition, or appearance of promotion, in the inhibiting zone may follow a fall in the concentration of ascorbic acid to a low level, with consequent loss of the 'protective' antioxidant effect, or at least be a refl ction of a changed oxidation-reduction status of the tuber. This effect of ascorbic acid has been suggested by Raadts and Söding (1947) and Raadts (1949). In short, the inhibiting zone apparently exhibits a balance between inhibiting and promoting effects, a balance which favours inhibition at the beginning of storage and possibly increasingly so during the first few months at 10° C. The balance then swings in favour of promotion or loss of inhibition but it is not necessary for this to occur before sprout growth can commence. In the four varieties studied it followed the onset of sprouting. The effect of temperature on this balance is shown by the samples of the variety Dr. McIntosh stored at 0° and 1° C. at which temperatures sprouting does not occur; 5° C. at which this variety shows signs of sprout growth, but the sprouts do not develop unless the storage temperature is raised: and 15° C. and 25° C. at which temperatures sprout growth is vigorous. It will again be noticed (Figure 6) that there is no obvious relationship between the activity of the inhibiting zone and sprout growth — over the 26 weeks of storage the extracts of tubers stored at 5° C. and at 15° C. behaved almost identically. At 0° and 1° C. there was a rapid loss of inhibition and appearance of promotion followed by a slower re-appearance of inhibition. At 25° C. there was a rapid increase in inhibition followed by a disappearance of the inhibiting effect. In this case sprout growth was occurring vigorously at a time when the inhibiting effect was at a maximum.

There is far too little evidence on which to suggest any possible connection between the effects of temperature upon growth substances and upon other constituents of the cell — as for example the effects upon ascorbic acid content discussed by Barker and Mapson (1950), upon the sugar balance (Müller-Thurgau, 1882: Barker, 1932, 1936) or upon the enzyme activity (Todd, 1953). It may be suggested however that it is difficult to find any obvious relationship between the effects of temperature upon growth substances and upon sprout growth.

Promoting zone. In the case of the four varieties stored at 10° C. there are, as in the case of the inhibiting zone, fluctuations in the effect of elutates of the promoting zone (Figure 5) but it would be difficult to relate these fluctuations precisely to sprout growth. The same applies to the effect of storage

temperature upon the activity of the promoting zone (Figure 6). Storage at
0° and 1° C. caused a rapid increase in the promoting effect followed by a
fall. At 5° C. there was a similar pattern of behaviour but the maximum was
higher and was reached later. At 15° C. there was little change, and at 25° C.
there was an initial fall followed by a rise. It seems possible that all the
activity might disappear after prolonged storage.

The final conclusion from the above work would seem to be that both
the promoting and inhibiting substances, in that they are products of the
metabolism of the cell, will mirror changes in conditions in the cell — such
as the loss during storage of the anti-oxidant effect of ascorbic acid, or an
increase or decrease of enzyme activity, or changes caused by different tem-
perature coefficients of related reactions. We may find in some instances
a tendency to a broad correlation between sprout growth and changes in
the growth substances, in that as the period of storage advances, sprouting
is tending to occur, inhibition is tending to decrease, and promotion is
tending to increase. It would be difficult to postulate any obligatory rela-
tionship however. A relationship could equally well be postulated between
sprouting and, say, the loss of ascorbic acid — which can act as a growth
inhibitor — or changes in the ascorbic acid/dehydroascorbic acid ratio (in
which connection see Raadts, 1949). This would, however, need to be recon-
ciled with the stimulation of sprouting by ascorbic acid as observed by Julén
(1944). The dual effect of ascorbic acid, noted by Havas (1935), is of interest
here. High concentrations (2.5—5 g./litre) inhibited the germination and
growth of wheat, while low concentrations (0.1—0.5 g./litre) had no effect
on germination but stimulated seedling growth. The inhibitory concentration
varied with the test material (perhaps because of its natural content of
ascorbic acid), being as low as 0.5 g./litre in the case of seedlings of tomato
and paprika.

The above remarks on growth substances apply specifically to ether extracts
prepared as described at the beginning of this paper, fractionated by adsorp-
tion on filter paper using water as the solvent, and tested by the Avena
straight growth method. Different methods of extraction would give a dif-
ferent selection of extractives — for instance a chromatogram run on What-
man No. 3 paper in water, prepared from an ether extract of the peel of the
variety Dr. McIntosh stored for 5 weeks at 5° C., and viewed under ultra-
violet light, showed a vivid light blue fluorescent spot at R_F 0.28, a green
fluorescent spot at R_F 0.44, a blue fluorescent spot at R_F 0.86 and a faint
dark absorbent spot at R_F 0.9. Similar treatment of a chromatogram from
a methanol extract of the same tubers showed a similar dark absorbent
spot, seven blue or greenish blue fluorescent spots between R_F 0.06 and R_F
0.74, no blue spot at R_F 0.86, no green spot at R_F 0.44, but a vivid bluish

green fluorescence at R_F 0.78. The different selections of substances which could be obtained by different methods of fractionation are clearly very many, and the assessment of a substance as growth promoting or inhibiting depends upon the method of assay — for instance scopoletin cannot be shown to be a growth inhibitor by the Avena straight growth method at concentrations up to about 51 mg./l., but it has been shown by Goodwin and Taves (1950) to be a very effective inhibitor of the growth of Avena roots at a concentration of about 6 mg./l. Irrespective of the methods of extraction, fractionation and assay, I feel that if over-riding significance is to be attributed to any substance with respect to the break of dormancy (and it is by no means certain that any one substance or reaction will be found to be of over-riding significance) it is not sufficient to establish a broad correlation between the presence or absence of this substance and sprout growth in a few instances. The correlation must be precise and widespread. Moreover, the substance must be proved to influence the growth of potato sprouts. If an inhibiting substance is to be postulated as the cause of dormancy, then that inhibiting substance must be shown to inhibit, not the elongation of Avena coleoptiles, but the growth of potato sprouts. Our own tentative experiments in this direction have been mentioned above, in which extracts of potato which reduce coleoptile elongation have been found to have but little effect upon the growth of potato adventitious roots. As was pointed out above, this method of assay, employing adventitious roots, is not really satisfactory, and a more relevant method must be developed. An adaption of the first three of Koch's four postulates would seem to be applicable to a study of the function of growth substances.

Attempts at identification of inhibiting substances. There have been no positive results from our attempts at identification. As was stated above, the zones as separated by us probably contain a mixture of substances. For instance the 'inhibiting' zone might contain a variety of substances such as *trans*-cinnamic acid (R_F 0.91 on Whatman No. 3 paper) and β-indolyl acetic acid (R_F 0.87). Although it might, at first sight, be tempting to suggest that the absorbent spot under u.v. at R_F 0.9, and the blue fluorescence at R_F 0.86, mentioned above as being present in ether extracts, might be *trans*-cinnamic acid and β-indolyl acetic acid respectively, there is no evidence for this. It has not proved possible to induce promotion in the inhibiting zone by irradiation with u.v. light, nor have any chemical tests for β-indolyl acetic acid given positive results.

In that there has been a tendency on the part of some workers to suggest the identity of growth substances on the basis of their R_F's in no great number of solvents it may be worthwhile here to recount our experiments with scopoletin. The most conspicuous substance revealed by a paper chro-

matogram, prepared from an ether extract[1] of the peel of a potato tuber, exhibits a vivid blue fluorescence in ultraviolet light, changing to greenish blue in the presence of ammonia vapour. When run on Whatman No. 1 paper at $20°$ C it had an R_F of 0.07 in ethyl acetate-2 N. ammonia $(1:1)$; of 0.59 in n-propanol-35 % ammonia-water $(10:1:1)$; and of 0.94 in m-cresol-acetic acid-water $(50:2:48)$. The observed R_F values, the colour of the fluorescence, the green coloration produced by spraying the chromatogram with 1 per cent aqueous potassium permanganate, and the brown coloration given by spraying with an acetone solution of silver nitrate, followed by sodium hydroxide and clearing with glacial acetic acid, all indicated that the fluorescent substance might be scopoletin (7-hydroxy-6-methoxy-coumarin), which has very similar properties (Swain, 1953). When mixed with a sample of scopoletin,[2] and run on filter paper in n-propanol-35 % ammonia $(7:3)$, and in m-cresol-acetic acid-water, the two substances ran as one. It was thought that the scopoletin, thus provisionally identified, might play a part in regulating the dormancy of the tuber, since unsaturated lactones are known to inhibit plant growth (Veldstra and Havinga, 1945) and of the coumarin derivatives tested by Goodwin and Taves (1950), scopoletin was found to have the greatest initial inhibiting effect, at low concentrations, upon the growth of Avena roots, although the roots recovered from the effect. We therefore tested the inhibiting effect of elutates of the fluorescent substance upon Avena coleoptile elongation. The substance was eluted from chromatograms run in ethyl acetate-water, $(R_F\ 0.89)$; ethyl acetate-2N.NH$_3$ $(1:1)$, $(R_F\ 0.0—0.2)$; and iso-propanol-water $(2:3)$ $(R_F\ 0.81)$. In every case the elutate had an inhibiting effect. This effect was also exhibited by the fluorescent zone eluted from a Dowex II column with methanol. Pure scopoletin, in concentrations up to 51 mg./l. was however found to have no significant effect upon Avena coleoptile elongation tested both by the straight growth method and by the curvature test; nor did it reduce appreciably the stimulation exerted by 0.08 mg./l. β-indolyl acetic acid. It was then found that the fluorescent substance and the inhibiting substance or substances in potato extracts could be separated by adsorption chromatography on paper using water as solvent. Elutates of the fluorescent substance from such chromatograms (using Whatman No. 3 paper), like

---

[1] The extracts used in the chromatographic investigation reported here were prepared from dormant tubers of the variety Stormont Dawn grown in 1952. Their concentration of what appeared to be scopoletin was very high — the cut surface of a tuber fluoresced vividly in ultra-violet light. A high concentration of scopoletin has been said to be correlated with leaf-roll infection and I therefore planted a quantity of these tubers in 1953. There was no sign of secondary leaf-roll in the resultant crop.

[2] Kindly supplied by Dr. T. Swain of the Low Temperature Research Station, Cambridge.

pure scopoletin, showed no activity in the Avena test. Later observations showed that break of dormancy occurred without marked loss of the fluorescent substance. The results of our work on scopoletin were thus negative, but they are of interest as illustrating the danger of basing an identification on R_F's, and as providing (if our provisional identification as scopoletin was correct) a further example of the possible danger of assuming a function of a substance in one species or organ (such as potato sprouts) on the basis of observations on another (such as Avena roots) — as mentioned above. sprouting occurred when the concentration of fluorescent substance was still high.

Summary

Although substances which inhibited and promoted Avena coleoptile elongation could be shown to be present in ether extracts of potato peel, it was not possible, on the basis of the work here reported, to demonstrate any obligatory relationship between the presence or concentration of these substances and the onset of sprouting. The effects of variety and temperature of storage upon the content of the substances were not obviously in accord with the effects of these variables upon sprouting. It is suggested that, if an inhibiting substance is to be postulated as the cause of dormancy in potatoes, the substance must be shown to inhibit the growth of potato sprouts. It is not sufficient, nor necessarily relevant, for it to be shown to inhibit the elongation of Avena coleoptiles. In general, if any particular physiological function is to be ascribed to a substance, the test material should be the species under investigation.

References

Allison, R. M. & Driver, C. M.: The effect of variety, storage and locality on the ascorbic acid content of the potato tuber. — J. Sci. Food Agric. 4: 386. 1953.

Appleman, C. O.: Special growth-promoting substances and correlation. — Science. 48: 319. 1918.

Ball, N. G.: The effects of certain growth-regulating substances on the rhizomes of Aegopodium podagraria. — J. exptl. Bot. 4: 349. 1953.

Barker, J.: Temperature and the metabolic balance in the potato. — Rep. Food Invest. Bd., Lond. for 1931, p. 86. 1932.

—   Analytic studies in plant respiration. VI. — The relation of the respiration of potatoes to the concentration of sugars and to the accumulation of a depressant at low tem-

peratures. Part 3. — The relation of the respiration to the concentration of sucrose. — Proc. Roy. Soc. B. 119: 453. 1936.

—   & Mapson, L. W.: The ascorbic acid content of potato tubers. II. The influence of the temperature of storage. — New Phytol. 49: 283. 1950.

Boysen Jensen, P.: Quantitative Bestimmung der beschleunigenden Streckungswuchsstoffe in der sauren Fraktion der Ätherextrakte aus höheren Pflanzen. — Planta 31: 653. 1941.

Goodwin, R. H. & Taves, C.: The effect of coumarin derivatives on the growth of Avena roots. — Amer. J. Bot. 37: 224. 1950.

Guthrie, J. D.: Control of bud growth and initiation of roots at the cut surfaces of potato tubers with growth-regulating substances. — Contr. Boyce Thompson Inst. 11: 29. 1939.

Havas, L.: Ascorbic acid (Vitamin C) and the germination and growth of seedlings. — Nature, Lond., 136: 435. 1935.

Hemberg, T.: Änderungen des Auxingehalts in Kartoffelknollen vor und nach dem Keimen. — Svensk Bot. Tidskrift, 36: 467. 1942.

—   Über das Vorkommen wachstumhemmender Stoffe in Kartoffelknollen und die Bildung wachstumfördernder Stoffe in Wundflächen derselben. — Arkiv för Botanik, 30 B, (7). 1943.

—   Weitere Untersuchungen über wachstumfördernde Stoffe in Wundflächen von Kartoffelknollen. — Svensk Bot. Tidskrift, 38: 428. 1944.

—   Wachstumhemmende und wachstumfördernde Stoffe bei der Kartoffel. — Arkiv för Botanik, 33 B, (2). 1946.

—   Studies of auxins and growth-inhibiting substances in the potato tuber and their significance with regard to its rest period. — Acta Horti Bergiani, 14: 133. 1947.

—   Significance of growth-inhibiting substances and auxins for the rest period of the potato tuber. — Physiol. Plant., 2: 24. 1949.

—   The effect of glutathione on the growth-inhibiting substances in resting potato tubers. — Physiol. Plant., 3: 17. 1950.

—   Establishment of acid growth-inhibiting substances in plant extracts containing auxins by means of the Avena test. — Physiol. Plant., 4: 437. 1951. ·

—   The significance of the acid growth inhibiting substances for the rest-period of the potato tuber. — Physiol. Plant., 5: 115. 1952.

—   Studies on the occurrence of free and bound auxins and of growth inhibiting substances in the potato tuber. — Physiol. Plant., 7: 312. 1954.

Julén, G.: Vitamin C metabolism in potatoes. — Lantbrukshögskolans Annaler. 12: 131. 1944.

Larsen, P.: Avena curvatures produced by mixtures of growth promoting and growth retarding substances. — Amer. J. Bot. 34: 349. 1947.

—   On the separation of acidic and non-acidic auxins. — Physiol. Plant., 8: 343. 1955.

Mes, M. G. & Menge, I.: Potato shoot and tuber cultures in vitro. — Physiol. Plant., 7: 637. 1954.

Michener, H. D.: Dormancy and apical dominance in potato tubers. — Amer. J. Bot. 29: 558. 1942.

Müller-Thurgau, H.: Ueber Zuckeranhäufung in Pflanzentheilen in Folge niedriger Temperatur. — Landw. Jb. 11: 751. 1882.

Raadts, E.: Über den Einfluss der Askorbinsäure auf die Auxinaktivierung. — Planta 36: 10. 1949.

—   & Söding, H.: Über den Einfluss der Askorbinsäure auf die Auxinaktivierung. — Naturwissenschaften 34: 344. 1947.

Skoog, F., Schneider, C. L. & Malan, P.: Interactions of auxins in growth and inhibition. — Amer. J. Bot. 29: 568. 1942.

Swain, T.: The identification of coumarins and related compounds by filter paper chromatography. — Biochem. J. 53: 200. 1953.

Todd, G. W.: Enzyme studies on dormant and active potato tubers. — Physiol. Plant.: 6: 169. 1953.

Veldstra, H. & Havinga, E.: On the physiological activity of unsaturated lactones. — Enzymologia, 11: 373. 1945.

Wildman, S. G. & Muir, R. M.: Observations on the mechanism of auxin formation in plant tissues. — Plant Physiol. 24: 84. 1949.

Zimmerman, P. W. & Hitchcock, A. E.: Activation of cinnamic acid by ultra-violet light and the physiological activity of its emanations. — Cont. Boyce Thompson Inst. 10: 197. 1939.

Auxin and Sexuality in *Cannabis sativa*

By

J. HESLOP-HARRISON

Department of Botany, Queen's University, Belfast
(Received June 12, 1956)

Introduction

It is now well established that sex expression in various monoecious Cucurbitaceae may be modified by auxin treatment. Laibach and Kribben (1950 a, 1950 b, 1950 c and 1950 d) have shown that the proportion of female flowers produced by the cucumber (*Cucumis sativus*) may be substantially increased by treatment during early growth with β-indoleacetic acid (IAA) and α-naphthaleneacetic acid (NAA), applied either in lanolin paste medium or by spraying in aqueous solution. Nitsch, Kurtz, Liverman and Went (1952) have reported a similar result with squash (*Cucurbita pepo* var. Table Queen), in which spraying with NAA in aqueous solution at 100 p.p.m. at the two-leaved stage induced the formation of the first female flower bud at about the ninth node, the first female flowers in control plants not being formed until the twentieth node or later. Corresponding results have been obtained by Wittwer and Hillyer (1954) with the cucumber varieties National Pickling and Burpee Hybrid, as well as with squash.

Arguing from their findings with cucumber, Laibach and Kribben (1950 d) have suggested that the sexuality of flowers is dependent upon the concentration of native auxin available in the leaf axil during the period of flower formation. Were this to be established as generally true for the flowering plants, it would constitute an important step forward in our understanding of the control of flowering and flower morphogenesis. Whilst the results so far reported for the monoecious cucurbits are themselves unequivocal, it would obviously be valuable to have a demonstration that auxin

levels govern flower sexuality in dioecious plants also. The present paper provides evidence that this may indeed be so in the dioecious species *Cannabis sativa* (hemp).

Materials and Methods

The material. The hemp strain used originated from the Botanic Garden of Coimbra, Portugal, and was inbred for two generations before being employed in the present experiments. This strain, as is normal in hemp, produces a slight preponderance of males, but in normal daylength conditions no plants have appeared showing monoecism or other sexual irregularities. Intersexuality normally appears in young male plants exposed to continuous short-day treatment (cf. Tournois, 1914; Schaffner, 1921 and 1923), but this effect is only apparent when night temperature falls below 16° C. In the present experiments night temperature was regulated between 20—22° C, and intersexuality appeared in none of the control plants.

Culture conditions. The seeds were germinated in vermiculite and the seedlings transplanted at a height of 2—3 cm. to individual pots containing a mixture of equal parts of washed sand and sphagnum peat, where they were supplied on alternate days with 10 ml. of a nutrient solution containing 150 p.p.m. KNO_3, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and 600 p.p.m. $Ca(NO_3)_2 \cdot 4H_2O$. Watering was carried out with tap-water twice daily.

The plants were grown throughout in a greenhouse with night temperature regulated between 20—22° C, and a mean maximum day temperature of 34.6° C (range 22—46° during the period of the experiments).

During short-day treatment, the plants were placed in dark cabinets from 5 p.m. to 9 a.m. daily, the circulation of air between the cabinets and the greenhouse being maintained by fans. During long-day treatment, they were exposed to natural days (varying between 12.5—15 hrs. during the experiments) with supplementary illumination to give a total light period of 21—22 hrs. This additional light was provided by high-pressure mercury vapour 'plant irradiators' of G E.C. manufacture which were suspended at varying heights to give 3,500—4 000 lux at the levels of the growing points.

Experimental methods. Since the intention was to establish in genetically male plants a supra-normal level of auxin in the axillary buds at the time of differentiation of flower primordia, it was apparent that both dosage and time of application were likely to be critical.

Advantage was taken of the photoperiodic sensitivity of hemp (a short-day plant, Tournois, 1914) to control the time of flower bud differentiation. The experimental plants were grown to an age of 20 days in long days, and then given an inductive treatment of ten short days. From the experience of previous experiments it was known that although flower buds are not usually visible after this treatment, they are differentiated actively during the ten or fifteen days following return to long days.

Also in preliminary experiments, it was found that NAA and IAA applied in aqueous solution at 100 p.p.m. by spraying the whole plant would totally suppress flowering after a period of photoperiodic induction so short as ten days. Furthermore, following the application of auxins in this way, hemp plants react violently

and erratically by stem curvature and occasionally by the later production of distorted leaves. Greater control over dosage was ultimately achieved by administering the auxins in a hydrous lanolin medium. In the Series A reported on below, NAA was applied in this manner in a concentration of 0.5 per cent. About 0.25 gm. of the paste was applied to the lower surface of the central lobe of one of the trifoliate leaves at the third node immediately following the cessation of short-day treatment, and similar quantities were applied to the central lobes of both leaves at node 4 five days later. The treated lobes were excised three days after the application of the paste in each instance.

The maximum number of plants that could be handled with the desired degree of uniformity in a single experimental series was sixteen. Series A (sixteen plants) was given short-day induction and auxin treatment as described above; Series B (fifteen plants), the same short day induction but no auxin, and Series C (ten plants) was maintained under long days throughout the first part of the experiment.

Since the genetical sex of a plant cannot be determined in the seedling stage, the distribution of the sexes in these groups was random, and unknown at the outset. To determine the genetical sex, all plants were brought into flower by continuous short-day treatment from the seventieth day of the experiment, i.e., forty days from the end of the ten-short-day induction treatment given to the plants of Series A and B. The final records of flowering state, phyllotaxis and leaf shape were taken immediately before this transfer to short days.

The distribution of the genetical sexes in the various groups proved to be as follows: Series A, eight males and eight females; Series B, five males and ten females; Series C, four males and six females.

Results

Sex expression

The flowers of hemp are produced in axillary spikes (♀) or racemes (♂) of greater or lesser degree of extension. The first-formed pair of flowers are produced on either side of the base of the axillary axes (Figure 1), lying within the stipules; their vasculation arises directly from the nodal system of the main stem, and they are perhaps therefore not to be regarded as part of the main axillary inflorescence. The sexuality of these basal flowers was recorded in the various experimental series. With the use of a low-power binocular microscope it is readily possible to distinguish fully developed male and female flowers *in situ*, the former possessing a 5-partite perianth within which the anthers can be observed some time before their dehiscence, and the latter having a small cup-like perianth of one member and an ensheathing bract, from the tip of which the stigmas project. Occasionally minute flower primordia are encountered which cannot be sexed, and these were not recorded. Some flowers formed after minimal photoperiodic induction are shed before maturity, the males before dehiscence of the anthers, and the females before the exsertion of the stigmas. Where it was possible

Table 1. Sexuality of flowers at successive nodes of the plants of series A and series B (plants...)

Series A

Plant no.	Genetical sex
H 1	male
H 2	male
H 3	male
H 4	male
H 5	male
H 8	male
H 9	male
H 15	male
H 6	female
H 7	female
H 10	female
H 11	female
H 12	female
H 13	female
H 14	female
H 16	female

Series B

Plant no.	Genetical sex
H 23	male
H 27	male
H 28	male
H 18	male
H 32	male
H 17	female
H 19	female
H 21	female
H 22	female
H 24	female
H 25	female
H 26	female
H 29	female
H 30	female
H 31	female

Node number: 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

V, flower primordia indistinguishable, or minute and unsexable.

♀+, normal female flower with extruded stigmas (cf. fig. 1 A).

(♀+), flowers recognised as morphologically female when dissected, but not developed to the point of stigma extrusion, or shed before this stage.

♂, normal male with anthers dehiscing in the usual manner (cf. fig. 1 B).

(♂), flowers recognised as morphologically male when dissected, but not developed to the point of anther dehiscence, or shed before this stage.

Plants scored up to node 17 or 18 in the males and 14 or 15 in the females just before final transfer to short days, and ten days later up to node 24 in the males and 20 in the females. The vertical bar indicates the transition to alternate from opposite phyllotaxis.

Figure 1. *The flowering behaviour of the genetically male plant H4 of series A.*
A. Female flowers produced at nodes 9 and 10. The flowers lie at either side of the base of the lateral shoot, and the ensheathing bracts and extruding stigmas may be dis-tinguished. The two flowers at node 9 are bicarpellary, those at node 10 tricarpellary but otherwise normal.
B. Male flowers at node 21 of this plant formed during the second period of flowering.

to establish the sex of these by dissection, they were recorded appropriately, but as 'aborted'.

The sex of flowers produced at successive nodes of Series A and B is recorded in Table 1. The plants of these series are arranged in groups according to the sex expressed in the final period of flowering following the second photoperiodic induction, this being regarded as their 'genetical' sex. The record for each node is based upon the most fully developed flower there. The appearance of female flowers produced on genetically male plants of Series A is shown in fig. 1 A, and of the male flowers later produced on the same plants, in Figure 1 B.

The following additional observations are of importance: (a) No male flowers at all appeared in any plant of the NAA treated Series A during the first period of flowering. The female flowers formed were structurally nor-mal, both in the genetically male plants and in the females, although a relatively high proportion were shed, or withered before the extrusion of the stigmas. In no instance in this series were flowers produced on the axillary axes, none of which extended appreciably until after the second
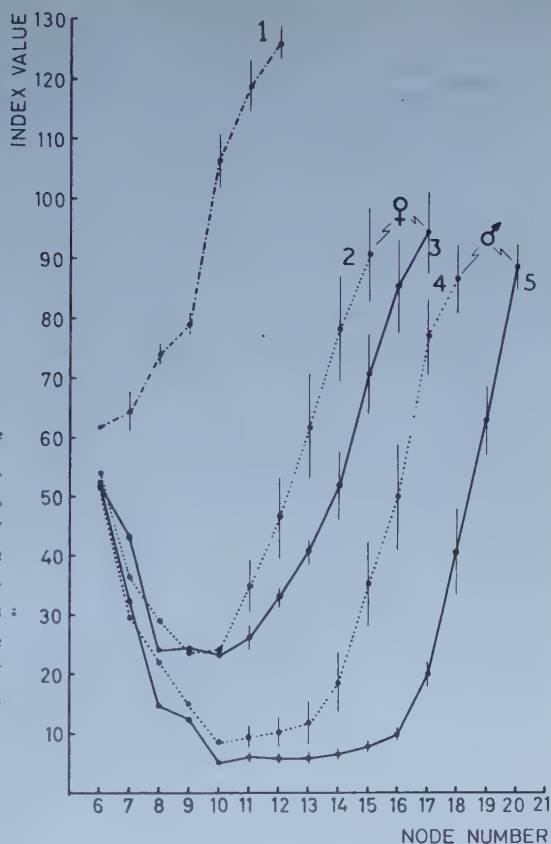
Figure 2. *The variation of the leaf shape index at successive nodes.* Curve 1, long-day controls; curve 2, female plants of Series A (short-day +NAA treatment); curve 3, female plants of Series B (short-day controls); curve 4, male plants of Series A (short-day+NAA treatment); curve 5, male plants of Series B (short-day controls). The vertical bars from node 7, curve 1, and from node 11 of the remainder correspond to 2 X Standard Error.

period of photoperiodic induction. The bracts enclosing the female flowers in both genetically male and female plants in this series showed some indication of hypertrophy as compared with the controls of Series B.

(b) Intersexuality did not appear in either male or female plants of the Series B in the first period of flowering. These plants produced moderately extended lateral inflorescences with normally sexed flowers.

(c) Up to the time of the final transfer to short photoperiods, the plants of the long-day control group, Series C, showed no macroscopic flowers. Dissection of the upper nodes showed the presence of minute flower primordia on a few of the plants which later flowered female following photoperiodic induction, but these primordia were unsexable.

Phyllotaxis

It is usual for hemp to commence with an opposite and decussate leaf arrangement, and then to pass over to alternate phyllotaxis during the period

of flowering (Figure 1). The point of this transition was regularly node
eight or nine in the plants of both Series A and B, and is indicated for each
plant by a vertical bar in Table 1. In the long-day Series C, no plant showed
a transition to an alternate leaf arrangement before the eighteenth node.

Leaf shape

Hemp shows a progressive change in leaf shape during development, the
transition to flowering being marked by a reduction in the number of leaf
lobes formed at successive nodes as well as by a decrease in the numbers
of marginal teeth on these lobes. Plants passing through a phase of flowering
— having, for example, been returned to long days after a period in short
days — show the reverse trend, a progressive increase in leaf lobe number
and serration number. These characteristics were recorded for all experi-
mental series. To give an over-all picture of leaf pattern, an index has been
compounded consisting of (lobe number) X (number of teeth on one margin
of the central lobe). The nodal sequence in this index in the different groups
is shown in Figure 2. In these graphs, the sexes have been kept separate in
Series A and B, but not in Series C, in which the sexes showed no appreciable
differences during the first 70 days of the experiment.

Discussion

From the flowering behaviour of the genetically male plants of Series A
as recorded in Table 1, there seems no reasonable doubt that the administra-
tion of NAA following photoperiodic induction can lead to the formation
of female flowers in sites which would normally be occupied by males. It
should perhaps be emphasised that this rather positive form of sex 'inversion'
is seemingly not directly comparable with the intersexuality which can be
induced in male plants of hemp by continuous short-day treatment, known
since the work of Tournois (1914) and Schaffner (1921, 1923). In the latter
form of intersexuality, male flowers produced in the characteristic manner
in axillary racemes contain organs showing various degrees of intergradation
between stamens and carpels, the first manifestation being the formation
of stamens with terminal stigmas. It is rare indeed to find morphologically
perfect female flowers in such plants, and in the writer's experience never
do they occupy the basal position in the axil.

If the obvious interpretation of the results obtained in the present experi-
ments is the correct one, then it seems reasonable to suppose that the
establishment of a high auxin level in the leaf axils just at the time of

differentiation of the flower primordia has suppressed the development of male flowers and provoked instead the formation of females. This is in complete agreement with the findings of Laibach and Kribben with Cucumis, and leads to the presumption that it is the level of native auxin in the vicinity of differentiating flower primordia which determines sexuality.

The simplest hypothesis (Heslop-Harrison, 1956) is that in this context auxin is acting as in its other regulatory activities in the plant, according to an 'optimum' curve (cf. Thimann, 1937), the optimum for staminate development being lower than that for pistillate, so that levels which promote the former suppress the latter, and *vice versa*. It perhaps needs no emphasis that such an activity *is* no more than regulatory; if it is indeed auxin level at the differentiating apex which determines the sexuality of the flowers produced, then we must suppose that it does so by modifying a pattern of organ formation which, in detail, is determined by the genetical make-up of the reacting tissue. Nevertheless, this demonstration that sexuality may be modified experimentally by manipulating auxin levels in a dioecious species is not without its implications for the general matter of genetical sex determination in plants.

A secondary aspect of the effect of NAA applied in this manner following photoperiodic induction is the very considerable reduction in the total flowering response. This effect is seen in Table 1 in the difference between Series A and Series B in the numbers of nodes at which fully developed flowers are formed, but this is by no means the complete picture, since axillary racemes were not formed at all in Series A, whilst in Series B they were formed at all of the flowering nodes except at the lowermost and uppermost. The suppressive effect of auxin on the flowering reaction in short day plants has been known since the work of Bonner and Thurlow (1949), and, as recorded above, flowering may be totally suppressed in minimally photoperiodically induced hemp by general sprays of the auxins IAA and NAA at 100 p.p.m. in aqueous solution.

What is of particular interest in the present experiment is the fact that not only does NAA affect the flowering response, but apparently also the leaf-shape changes which accompany it. This is evident from the curves for the leaf-shape index in Figure 2. The long-day control series show a steadily increasing value throughout the two months of the experiment, the final leaves formed having seven lobes and an average of twenty teeth on each margin of the largest. In Series B, short-day induced without auxin, a marked difference between the sexes is evident. The females fall to an index value around 25 (three lobes, about eight teeth) by node 8 at the beginning of alternate phyllotaxis where flowering is copious, and begin to recover at node 11 as the plants pass through flowering, rising to a value of about 95

(seven lobes, thirteen or fourteen teeth) at node 17 after two months. Beginning with the same average index value, the males fall to a value of 5 or 6 (one lobe, five or six teeth) by node 10, and do not begin to recover until node 15 or 16, reaching a value of about 89 by node 20. The difference between males and females becomes statistically significant at node 10. In Series A, photoperiodically induced and then auxin treated, the sexes again show different behaviour, the trend being the same for Series A and B females up to node 11, and for series A and B males up to node 14. Thereafter the auxin-treated series in both sexes show a more rapid recovery to high index values, the difference between Series A and Series B being significant from node 12 and onwards in the females, and node 15 and onwards in the males. Notwithstanding this more rapid recovery, fewer leaves were unfolded in the male and female plants of Series A than in those of corresponding sex in Series B during the first two months of the experiment.

Summary

The effect of α-naphthaleneacetic acid on the sexuality of the dioecious *Cannabis sativa* (hemp) has been investigated in plants flowering after a period of minimal photoperiodic induction. In genetically 'male' plants, female flowers were formed in sites which would normally be occupied by males, a result which appears to provide evidence that flower sexuality may be normally regulated by the level of native auxin in the vicinity of the meristems during the period of differentiation of flower primordia. Secondary effects of auxin treatment are seen in an over-all reduction in the intensity of the flowering response, and in the modification of the course of heteroblastic development, the trend towards a reduction of leaf lobing and serration which normally accompanies flowering being reversed sooner in NAA treated plants passing through a period of flowering than in untreated controls.

References

Bonner, J. & Thurlow, J.: Inhibition of photoperiodic induction in Xanthium by applied auxin. — Bot. Gaz. 110: 613. 1949.
Heslop-Harrison, J.: The experimental modification of sex expression in flowering plants. — Biol. Rev. (in press) 1956.

Laibach, F. & Kribben. F. J.: Der Einfluss von Wuchsstoff auf die Bildung männlicher und weiblicher Blüten bei einer monözischen Pflanze (Cucumis sativa L.). — Ber. Deutsch. Bot. Ges. 62: 53. 1950 a.

— — Der Einfluss von Wuchsstoff auf das Geschlecht der Bluten bei einer monözischen Pflanze. — Beiträge Biol. Pflanz. 28: 64. 1950 b.

— — Der Einfluss von Wuchsstoff auf die Blütenbildung der Gurke. — Naturwiss. 37: 114. 1950 c.

— — Die Bedeutung des Wuchsstoffs für die Bildung und Geschlechtsbestimmung der Blüten. — Beiträge Biol. Pflanz. 28: 131. 1950 d.

Nitsch, J. P., Kurtz, E. B., Liverman, J. L. & Went, F. W.: The development of sex expression in Cucurbit flowers. — Amer. J. Bot. 39: 32. 1952.

Schaffner, J. H.: Influence of environment on sex expression in hemp. — Bot. Gaz. 71: 197. 1921.

— The influence of relative length of daylight on the reversal of sex in hemp. — Ecology 4: 323. 1923.

Thimann, K. V.. On the nature of the inhibitions caused by auxin. — Amer. J. Bot. 24: 407. 1937.

Tournois, J.: Études sur la sexualité du houblon. — Ann. Sci. Nat. Bot. 19: 49. 1914.

Wittwer, S. H., & Hillyer, I. G.: Chemical induction of male sterility. — Science 120: 893. 1954.

The Basis of Drought Resistance in the Soybean Plant

By

Jasper A. Clark and J. Levitt

Botany Department, University of Missouri
(Received June 14, 1956)

Environment resistance (e.g. drought, frost, or heat resistance) may be due to either avoidance or tolerance (Levitt 1956). In the case of frost, resistance is nearly always due to tolerance. In the case of drought, both avoidance and tolerance are important; in fact, the major differences that have been found between species are those that lead to drought avoidance — e.g. thick cuticle, deep root systems, etc. On the other hand, the adaptation (or "hardening") to drought as a result of preexposure to moderate drought has not been adequately investigated.

Clements (1937) showed that the soybean does become adapted to drought. He noticed that wilted plants gradually recovered their turgor without any improvement in their water supply. Correlated with this adaptation was an increase in hemicellulose. On the basis of this and certain other observations, Clements suggested that changes within the protoplasm were responsible for the reduced water loss.

The purpose of the present investigation was to test Clements' explanation as well as the following two others:

a) New cell wall material (Clements' hemicellulose) is laid down by apposition (inside the old cell wall) and in this way permits the protoplast to retain its turgor with a smaller water content. The change in the cell wall would also lead to an increased elastic extensibility, so that the cell would have to lose a larger fraction of its water before becoming flaccid. Both of these factors would permit the leaves of droughted plants to remain turgid in spite of water losses that would result in wilting of nondroughted plants. This would, then, be a case of drought resistance due to tolerance.

b) The droughted plants have in some way developed a better method

of maintaining a suitable water balance, either by an increased absorption or by a decreased loss. This would be a case of drought resistance due to avoidance. Since the experimental plants in this investigation were grown in pots, the possible benefits of a larger root system such as might occur in the field, were largely eliminated. Consequently, drought avoidance of such plants could only be due to a reduced transpiration rate.

Methods

Seeds of *Soja max*, variety Chief, were planted in four inch pots in the greenhouse on May 20, 1952. All plants were well watered until June 11 on which date the pots were separated into two groups. The plants in group one (nondroughted) were watered once or twice a day as needed to prevent them from wilting. The plants in group two (droughted) were watered only after undergoing permanent wilting. The same procedure was used in the fall and winter of 1955—56. The plants usually had five nodes when separated into the differentially watered groups and only one plant was left in each pot. The time required from planting the seeds until separation of the plants into the two groups was about one month. Seeds were planted every two weeks during the experiments.

Elastic extensibility of cell walls was measured by determining the contraction of living cells due to osmotic loss of a standard degree of turgor. Petioles were placed in a 0.20 molar solution of calcium chloride which was a little less than the concentration of the sap of the cells to be measured. Intercellular air was then removed with an aspirator before longitudinal sections were made. The sections were stained in a 0.20 molar solution of calcium chloride to which neutral red had been added. Measurements were made only on stained and, therefore, living cells. The staining also insured identification of the same cells for successive measurements. The sections were placed in 0.25 molar calcium chloride solution for the original measurement of parenchyma cells. Then they were transferred consecutively into 0.20 M, 0.10 M, 0.05 M, and finally once again in to 0.25 M solutions. The difference between the lengths in the 0.05 M solution and in the last 0.25 M solution was taken as their elastic stretch. The percentage of elastic extensibility was obtained by the ratio of elastic stretch to the original length in the first 0.25 M calcium chloride solution.

Transpiration rates were measured both with whole shoots and excised leaves. In the former case the shoots were cut above ground, set in test tubes, and weighed from time to time. In the latter case, two balances were set up side by side. Comparable leaves from a droughted and a nondroughted plant were excised at the petioles, leaving the three leaflets attached to each other. Each leaf was hung by wires from the pan-hook of one of the balances. This permitted free evaporation from the surface of the leaves. Weights were recorded every fifteen minutes for two hours.

To eliminate the protection by the cuticle and any other epidermal variation between the droughted and nondroughted leaves, both types were cut in strips onetenth of an inch wide and then weighed in the same manner as the whole or uncut leaves. Graph paper ruled ten lines to the inch was placed over the leaves to facilitate uniform cutting of the leaves. Some of the other methods used can be best discussed in connection with the problems investigated.

Results

a) Elastic extensibility of the cell walls. It was soon observed that the results varied, depending on whether or not the adjacent cells at the two ends of the one being measured were alive. Another important factor was the position of the section — i.e. whether it was taken from the base or top of the petiole. But as long as these factors were kept constant for droughted and nondroughted plants, the results were the same (Table 1) — the cell walls of droughted plants were significantly less extensible than those of the nondroughted plants. Many more measurements were made, always with the same results (Clark 1956). This directly contradicts the first hypothesis stated above.

b) Transpiration. Though the plants were grown in pots instead of in the field as in Clements' experiments, the droughted ones showed the same ability to remain turgid under conditions leading to wilting of the non-droughted plants. The droughted plants also lost water less rapidly (Figure 1). Similar results were obtained when excised leaves were used, though the difference was completely removed and even reversed when the leaves were cut into strips (Figure 2). These experiments were repeated several times with the same results (Clark 1956). It is therefore apparent (1) that Clements' theory is incorrect; for the rate of water loss from the exposed cells of leaves cut in strips is even more rapid in the case of droughted than of undroughted plants. This fact also proves that (2) the reduced transpiration rate from

Table 1. *Percent elastic extensibility of parenchyma cells of the petioles of soybean plants. Cells measured were in contact with other vitally stained cells at both ends.*

Cell No.	Droughted soybean		Nondroughted soybean	
	Size in microns	% elastic stretch in length	Size in microns	% elastic stretch in length
1	90.2×66	9.8	107.8× 88.0	18.3
2	103.4×77	10.6	94.6× 94.6	25.6
3	110.0×72.6	12.0	105.6× 94.6	22.9
4	90.2×66	9.8	96.8× 90.2	25.0
5	103.4×77	8.5	107.8× 92.4	30.6
6	110.0×72.6	12.0	46.2× 72.6	33.3
7	88.0×77	10.0	74.8× 46.2	17.6
8	57.2×85.8	15.4	46.2× 99.0	28.6
9	70.4×88	6.2	57.2×110.0	23.1
10	46.2×79.2	9.5	48.4×103.4	27.3

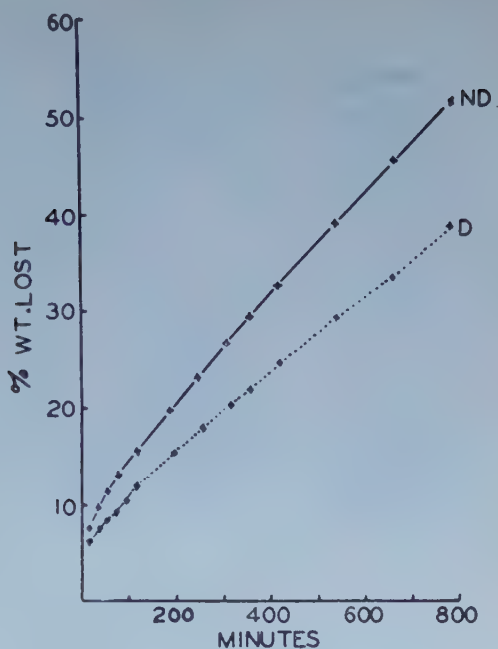|				
	mean percentage	$=10.38$	mean percentage	$=25.23$
	standard deviation	$= 2.428$	standard deviation	$= 5.01$
	standard error	$= .77$	standard error	$= 1.608$
	fiducial limits $(t_{.01})$		fiducial limits $(t_{.01})$	
		$l_1= 7.88$		$l_1=20.01$
		$l_2=12.88$		$l_2=30.45$

Figure 1. *Water loss* (as percent of fresh weight) *by excised shoots of droughted* (D) *and nondroghted* (ND) *soybean plants.*
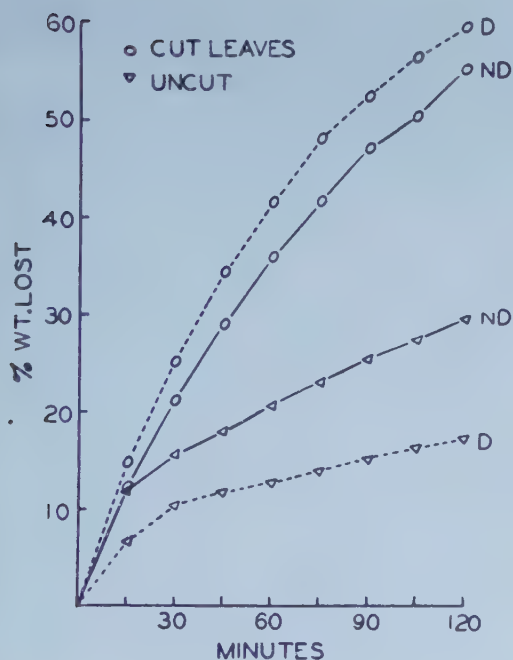

Figure 2. *Water loss* (as percent of fresh weight) *by excised leaves from correspond-ing nodes of droughted* (D) *and non-droughted* (ND) *soybean plants.* The blades used for the upper curves were cut into strips 0.1 inch wide.

Table 2. *Lipid removal with petroleum ether from comparable leaves of droughted and nondroughted plants.*

Treatment	Sq. cm. of leaf surface	mg. lipids removed	mg. lipids removed per sq. cm.	Lipid increase per sq. cm. in droughted leaves
Droughted...........	65.79	1.8	0.0273	
Nondroughted	62.89	1.1	0.0174	
				0.0099
Droughted	72.76	0.9	0.0123	
Nondroughted	78.05	0.0	0.0000	
				0.0123
Droughted	45.99	2.0	0.0434	
Nondroughted	51.34	1.2	0.0233	
				0.0201
Droughted	67.53	2.2	0.0325	
Nondroughted	71.92	1.6	0.0222	
				0.0103
Droughted	44.63	1.3	0.0291	
Nondroughted	72.69	1.5	0.0206	
				0.0085

leaves of droughted plants was due to the surface layer of the leaf. That it is not a stomatal control follows from the fact that the major difference occurs after the bend in the transpiration curve, when the rate rapidly decreases to a relatively small fraction of the original, due to stomatal closure (Stålfelt 1955). It must, therefore, be a cuticular control of transpiration — i.e. the cuticular transpiration is less rapid after droughting.

Such a decrease in cuticular transpiration might be caused by a deposit of a thicker layer of cuticular lipid as a result of droughting. Direct observation under the microscope failed to reveal any such difference. In fact, the cuticular layer could not be detected as such and must have been an integral part of the outer epidermal cell wall. It should be possible, however, to detect such a difference by extracting the leaf surface lipids and determining their quantity. This was first attempted by immersing the leaves in 1 : 1 alcohol-ether. But the solvent penetrated the leaves and extracted chlorophyll as well as the surface lipids and therefore could not be used. Petroleum ether did not become colored in two hours so it was used as the solvent.

The outline of each leaf was traced on paper and the area later was determined with a planimeter. After tracing the leaf it was placed in 100 ml. of petroleum ether for two hours. The solution was poured into weighing bottles and the ether evaporated. A control bottle with residue from 100 ml. of petroleum ether showed a gain of one milligram after the ether was evaporated. This correction was verified and then used as a correction factor for the results.
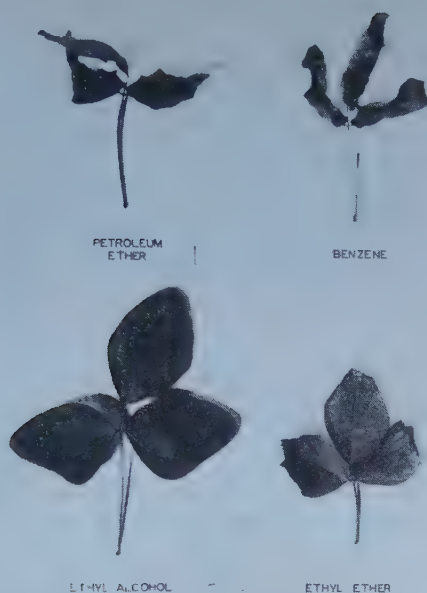
Figure 3. *Soybean leaves dipped in different solvents while still attached to the plant and excised 24 hrs. later.*

In all cases, the droughted leaves had more surface lipids per unit area than did the nondroughted leaves (Table 2). But is this difference sufficient to account for the difference in transpiration rates? Calculations indicate that it is, for the ratio between the relative amounts of surface lipids on leaves of droughted and nondroughted plants agrees well with the inverse ratio of the two transpiration rates (Table 3). As a direct, experimental test

Table 3. *Transpiration loss and lipids removed from unit areas of excised leaves from droughted and nondroughted soybeans.*

Exp. No.	Transpiration loss in gm. for $1\frac{1}{2}$ hrs. (starting $\frac{1}{2}$hr. after excision)				Lipids per cm^2 leaf surface (from Table 2)		
	ND	D	$\dfrac{D}{ND}$	$\dfrac{D}{ND} \times \dfrac{(1.236)\ \text{Area ND}}{\text{Area D}}$	ND	D	$\dfrac{ND}{D}$
1	.107	.052	.486		.0174	.0273	.637
2	.110	.061	.554		.0000	.0123	
3	.155	.074	.477		.0233	.0434	.536
4	.071	.035	.493		.0222	.0325	.638
5	.154	.068	.441		.0206	.0291	.707
aver.	.1194	.058	.486	.600	.0167	.0289	.577
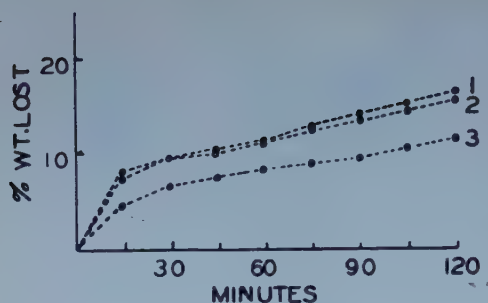
Figure 4. *Water loss* (as percent of fresh weight) *by excised leaves of droughted soybean plants.* Leaves 1 and 2 were dipped in alcohol 24 hrs. before removal from the plant. Leaf 3 was untreated.

of this conclusion, the attempt was made to remove the excess lipid on the surface of the droughted leaves and then determine the transpiration rate. This led to some difficulties, since the best lipid solvents injured the leaves (Figure 3). Alcohol, however, did succeed in removing some lipid (though less than the other solvents) without producing any injury. Such alcohol treated leaves when tested 24 hrs. after treatment no longer possessed the reduced transpiration of the untreated droughted leaves (Figure 4). In some cases, this method even increased their transpiration rates up to or beyond that of the untreated nondroughted leaves (Figure 5). These results agree with the calculations of Table 3, and indicate that the reduced transpiration rates of droughted leaves are completely accounted for by the increase in surface lipids.

The converse method was attempted without success. A solution of the leaf surface lipids when applied to the undroughted leaves failed to decrease their transpiration rates. On the contrary, the rates were actually increased. But the reason for this could easily be seen. Instead of simply depositing new lipid on the leaf, this method apparently redistributed the surface lipids leaving some areas with more lipid but others with less. As a result, the surface of the leaf showed irregular patches. A method of depositing new lipid without disturbing the old has not yet been worked out.

These results conclusively show that (1) the ability of droughted soybeans to remain turgid under conditions that lead to wilting of non-droughted soybeans is due to the reduced cuticular transpiration of the former; and that (2) this difference in transpiration rate is due to a deposit of more lipids per unit leaf surface in the case of the droughted plants. Consequently, the drought resistance of droughted soybeans is due to drought avoidance.

There is still the possibility that the droughted soybean may also possess drought tolerance due to dehydration hardiness (Levitt 1956). In order to

Figure 5. *Water loss* (as percent of fresh weight) *by excised leaves of droughted* (D) *and nondroughted* (ND) *soybean plants.* Plants droughted for 9 weeks (A), 4 weeks (B), and 2½ weeks (C). Leaves of droughted plants were dipped in alcohol 24 hrs. before excision.
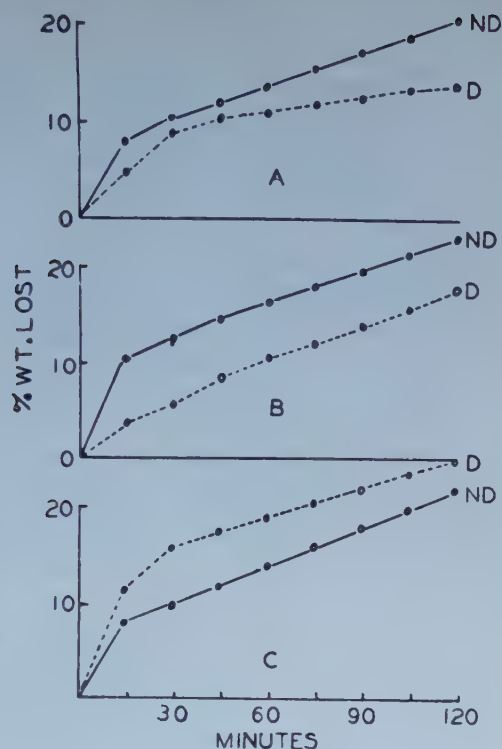
test this possibility, sections from the petioles of both droughted and non-droughted leaves were placed in a series of atmospheres of different relative humidities in order to determine the drought killing relative humidity (Levitt 1956). The results showed that droughting failed to change the dehydration hardiness (Table 4). It can, therefore, be concluded that the drought resistance of droughted soybeans is due solely to drought avoidance.

Table 4. *Killing of cells in petiole sections of droughted and nondroughted soybean plants in different relative humidities.*

Relative humidity	Percent of cells killed	
	D	ND
100	0	0
99	0	0
98	0	0
97	25	25
96	50	50
95	75	75
90	100	100

Summary

1. The cell walls of droughted soybean plants are less elastically extensible than those of nondroughted plants. This is the opposite of what would be expected from their ability to remain turgid longer.

2. The droughted plants transpire less rapidly than the nondroughted plants. But this difference is eliminated and even reversed when the protective effect of the surface is removed by cutting the leaves into strips.

3. The droughted plants possess more leaf surface lipids per unit area than the nondroughted plants. Removing some eliminates or even reverses the difference in transpiration.

4. There is no difference in dehydration hardiness between droughted and nondroughted plants.

5. The drought resistance of droughted soybean plants is solely drought avoidance, due to a deposit of more leaf surface lipids. Whether or not an increased root system could play a role in the field was not investigated, since the plants used were grown in pots.

References

1. Clark, Jasper A.: An investigation of the drought hardening of the soybean plant. — Ph. D. Thesis, U. of Missouri, 1956.
2. Clements, H. F.: Studies in drought resistance of the soybean. — Res. Studies of the State College of Wash. 5: 1. 1937.
3. Levitt, J.: The Hardiness of Plants. — Academic Press, New York 1956.
4. Stålfelt, M. G.: The stomata as a hydrophotic regulator of the water deficit of the plant. — Physiol. Plant. 8: 572. 1955.

The Effect of Catalase, Riboflavin and Light on the Oxidation of Indoleacetic Acid

By

E. R. WAYGOOD and G. A. MACLACHLAN

Department of Botany, University of Manitoba,
Winnipeg, Canada
(Received June 27, 1956)

Introduction

It is known that catalase can function as a peroxidase with various sub-
stances including alcohols, amines and phenolic compounds (Keilin and
Hartree. 12. Tauber, 21). In the presence of high concentrations of catalase
and a suitable hydrogen donor and with low concentrations of peroxide, the
catalatic decomposition of peroxide may become negligible and give way to
a peroxidase oxidation (Chance, 3, Keilin and Hartree, 12). Accordingly.
catalase would be expected to operate peroxidatically in systems that con-
tinuously generate and also utilise either organic or inorganic peroxide,
provided the catalase-peroxide complex is capable of reacting with the
hydrogen donor. A case in point is in the oxidation of indoleacetic acid.

The oxidative decarboxylation of the plant growth hormone indoleacetic
acid (IAA) is catalysed by peroxidase extracts from wheat leaves (Waygood
et al., 23, 24) and horse-radish roots (Kenten, 13). Our investigations to
date (Maclachlan and Waygood, 17, 18) have shown that while hydrogen
peroxide does not bring about the complete destruction of IAA yet the reac-
tion sequence most probably involves peroxidation of a specific organic
cofactor e.g. resorcinol or a monohydric phenol, by an intermediate organic
peroxide. No enzyme other than one having peroxidatic activity appears to
be required since the oxidised cofactor oxidises manganese as demonstrated
by Kenten and Mann (14) and manganic ions in turn initiate the spon-

taneous decarboxylation and oxidation of IAA (Maclachlan and Waygood, 16). The reaction is cyclic since the phenolic compound is reformed during manganese oxidation. The peroxidase substrate is considered to be a skatole peroxide (Maclachlan and Waygood, 17. Waygood *et al.*, 24, cf. Ray and Thimann, 19). The present communication deals with the ability of commercial beef liver catalase (Nutritional Biochemical Co. Ltd.) to substitute for peroxidase, and also with the antagonistic effects of riboflavin and light on the reaction.

Experimental Results

1. *Cofactor requirements*

Indoleacetic acid (2.22×10^{-3} M) was neither decarboxylated nor oxidised when incubated with 0.18 per cent dialysed catalase at pH 6.0 for three hours in standard respirometers. The presence of manganese (10^{-3} M) and resorcinol or monohydric phenols e.g. 2,4-dichlorophenol (5×10^{-4} M), separately were without effect on the system, but together with catalase brought about a rapid and complete oxidation of IAA (Table 1, Figure 1).

Carbon dioxide release determined by Warburg's direct method paralleled and slightly preceded oxygen uptake. The reaction was thermolabile and the optimum pH using 2,4-dichlorophenol (DCP) lay between pH 5.0 and pH 6.0. The end product of the reaction was a water-soluble yellow substance which possessed the same R_f value and crystallised from ethanol with the same crystal structure as the corresponding products from the non-

Table 1. *Effect of organic cofactors on IAA oxidation by catalase and peroxidase extracts.* System contains peroxidase (0.2 mgm.), catalase (5.5 mgm.) or wheat leaf extract (0.2 mgm. protein), manganese (3.0 µgm.), orthophosphate pH 6.0 (150 µgm), IAA (6.67 µgm = 158 µl) in a final volume of 3.0 ml. Cofactor concentrations as follows: dichlorophenol, 1.5 µgm; maleic hydrazide, 30 µgm; natural factor, 0.15 ml.; resorcinol, 1.5 µgm. Temperature 29.5° C.

System	Maximum velocity (µl. O_2/5 min.)				
Organic cofactor	Dichloro-phenol	Maleic Hydrazide	Natural Factor	Resorcinol	None
Beef liver catalase . . .	39.0	49.5	0	18.0	0
Wheat leaf extract . . .	44.5	25.5	17.5	31.5	6.0
Horseradish peroxidase	67.5	76.5	43.5	68.5	14.0
	Oxygen consumed at equilibrium (µl. O_2/3 hr.)				
Beef liver catalase . . .	157	150	0	86	0
Wheat leaf extract . . .	153	146	128	162	36
Horseradish peroxidase	136	153	126	149	92

Figure 1. *Effect of natural inhibitors and light on IAA oxidation by catalase.* System: as for Table 1 using catalase and dichlorophenol, IAA added at zero time.

A. control in darkness.
B. control in white light (1380 ft. candles).
C. control in darkness, boiled undialysed catalase (10 mgm. dry weight) added at arrow.
D. control in light, boiled undialysed catalase added at zero time.
E. same as D in darkness.
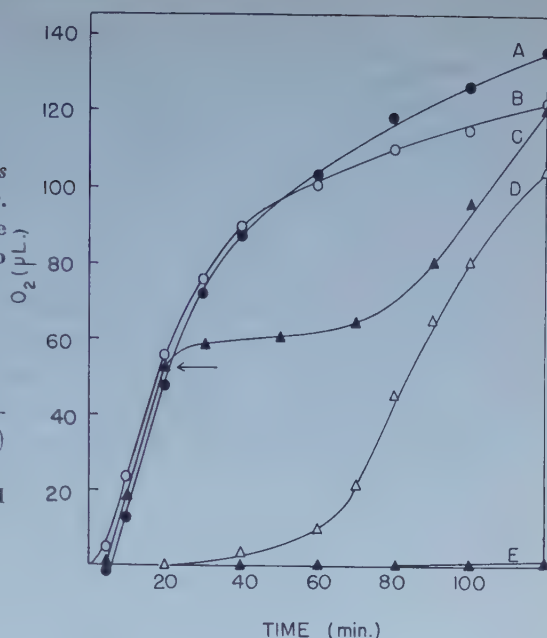
enzymic (Maclachlan and Waygood, 16) and peroxidase-catalysed reaction (Waygood *et al.*, 24).

The initial velocities *i.e.* the maximum velocity in any 10 min., and the absolute amounts of oxygen consumed by IAA after the attainment of equilibrium in the presence of either catalase, horseradish peroxidase, or wheat leaf extracts are compared in Table 1. The relative rates of IAA oxidation catalysed by peroxidase or catalase vary in the presence of different cofactors indicating reaction or complex formation between enzyme and cofactor. The growth inhibitor maleic hydrazide substituted for DCP in all systems, although a relatively higher concentration (10^{-2} M) was required to produce similar activity. In the catalase system with maleic hydrazide IAA oxidation occurred only after an induction period of 25 min., although in the peroxidase system with maleic hydrazide and in all systems containing DCP the induction phase lasted for only 1—3 min. The catalase system also differed from the peroxidase systems in that it was completely inactive towards IAA in the absence of an organic cofactor (Table 1). A naturally occurring cofactor which cannot be removed by extended dialysis appears to be associated with peroxidase. A crude ether-soluble fraction from wheat leaves (Waygood *et al.*, 24) substituted for the artificial cofactor in the wheat leaf and horseradish peroxidase systems, but was ineffective with catalase

(Table 1). This may indicate that the natural factor from wheat leaves reacts with peroxidase specifically.

Resorcinol at the same concentration as DCP (5×10^{-4} M) was equally effective in promoting IAA oxidation in the horseradish peroxidase system, but less effective in the wheat leaf system. On the other hand a 30 min. induction phase occurred in the resorcinol-catalase system, the rate was lower and less IAA was oxidised at equilibrium (Table 1). Further experiments revealed slight, but reproducible differences between catalase and other systems in the optima of pH and resorcinal concentrations. With 5×10^{-4} M resorcinol the optimum manganese concentration (succinate buffer) was 10^{-2} M with catalase, and 10^{-3} M with wheat leaf extract and horseradish peroxidase (Maclachlan and Waygood, 17). At suboptimum manganese concentrations for the catalase system (*i.e.* 10^{-3} M) the optimum resorcinol concentration was correspondingly lower (10^{-4} M). The ratio of the activities at pH 5.0 and pH 6.0 under optimum conditions of cofactor concentration was much greater in the catalase system (3.3) than in the wheat leaf system (1.4). Under conditions close to optimum for the catalase system (pH 5.0, 5×10^{-4} M resorcinol, 10^{-2} M manganese) resorcinol promoted the most rapid rate of IAA oxidation found in the entire catalase investigation (63 µl/5 min.).

Because of the quantitative differences between the catalase and peroxidase systems it is suggested that the activity of the wheat leaf system which contains both catalase and peroxidase is due to peroxidase chiefly. Further studies of the kinetics of these reactions would undoubtedly reveal other quantitative differences, nevertheless with respect to cofactor requirements the two systems are qualitatively identical.

2. *Enzyme concentration*

The peroxidatic activity of catalase appeared to be inactivated during IAA oxidation in the same way as wheat leaf extract (Maclachlan and Waygood, 17, Waygood *et al.*, 24) and horseradish peroxidase (Kenten, 13). With low catalase concentrations (< 0.05 per cent) the rate of oxygen uptake declined prematurely and resumed only if more catalase (or peroxidase) were added. When hydrogen peroxide was introduced to the flasks at this stage oxygen was evolved vigorously. Slightly more oxygen was evolved (114 per cent) from a system containing all the components with the exception of IAA, and less was evolved (90 per cent) from a solution of catalase only at the same concentration. It was concluded that during the oxidation of IAA the catalatic activity of catalase was retained whereas its peroxidatic activity was inhibited. Inactivation of both peroxidase and catalase is probably reversible and due to a strong binding of a reaction intermediate at peroxidatic sites.

In both the catalase and peroxidase systems the enzyme concentration/ activity curve is linear only at low concentrations of enzyme. At higher concentrations of catalase ($>$ 0.10 per cent) the rate of IAA oxidation approached a maximum. This has also been observed with other IAA 'oxidase' systems (Goldacre, 7, Goldacre and Galston, 8, Gortner and Kent, 10) and is discussed more fully elsewhere (Maclachlan and Waygood, 17).

3. *Inhibitors*

Part of the evidence for the presence and participation of manganic ions in the peroxidase-catalysed oxidation of IAA is the permanent inhibition which results from the addition of mangani-complexing agents to the system (Waygood *et al.,* 24). In a standard catalase-DCP system (Table 1), the addition of 2×10^{-3} M citrate or pyrophosphate prevented oxygen uptake and the formation of the yellow end product for a period of at least 24 hr. after the addition of IAA.

Undialysed catalase preparations inhibited IAA oxidation catalysed by wheat leaf extracts (Waygood *et al.,* 24) and were unable to catalyse IAA oxidation in the presence of optimum cofactor concentrations. The dialysable inhibitor(s) in crude catalase was thermostable since boiled undialysed catalase, when added prior to IAA, prevented its oxidation for at least 24 hr. in otherwise active systems (Figure 1 E). If, on the other hand, boiled undialysed catalase were added *after* the oxidation had commenced, its inhibitory effect was overcome in about one hour (Figure 1 C). Evidently the inhibitor interfered with a reaction which was essential both for the initiation of IAA oxidation and its propagation, but was destroyed by products formed during the oxidation.

Most artificial inhibitors are capable of acting as hydrogen donors in peroxidatic reactions by catalase or peroxidase, but these differ from the essential organic cofactors e.g. resorcinol, by being incapable in oxidised form of oxidising manganese. Inhibition by boiled undialysed catalase resembles that brought about by catechol in the wheat leaf system which was overcome by manganic ions (Waygood *et al.,* 24). Significantly, prior incubation of boiled undialysed catalase with manganiversene almost completely abolished its inhibitory effect. However, white light which had no effect on catechol inhibition (Waygood *et al.,* 24), decreased the lag period induced by boiled catalase to about one hour (Figure 1 D). Evidently the natural inhibitor in catalase unlike catechol absorbs visible light which facilitates its destruction. It is tempting to speculate that a metastable light-activated intermediate formed from the inhibitor causes an additional production of manganic ions which brings about its destruction. The inhibitor was removed with difficulty from catalase since the control system that had been dialysed
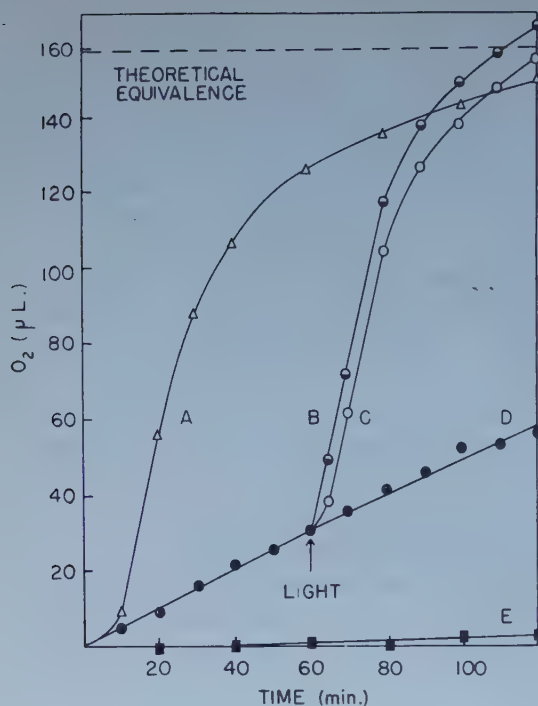
40

Figure 2. *The effect of riboflavin phosphate and light on IAA oxidation.* Catalase-dichlorophenol system as in Table 1.

A. control in darkness.
B. control$+10^{-4}$ M riboflavinphosphate. Illuminated with blue light (220 ft. candles) at 60 min.
C. same as B with white light (1380 ft. candles).
D. same as B with red light (960 ft. candles).
E. control in darkness$+5 \times 10^{-4}$ M riboflavinphosphate.

for 72 hr. still showed a short induction period (Figure 1 A) which was almost overcome by illumination (Figure 1 B). It is noteworthy that the rate of oxidation in light with or without inhibitor was no greater than the rate in darkness and therefore light cannot be regarded as an activator, but only as an agency overcoming natural inhibition in this case.

Besides the permanent inhibition caused by mangani-complexing agents and the temporary inhibition by substances which are destroyed by the system the oxidation of IAA by catalase may proceed without a lag period, but at a slower rate in the presence of 'retarders'. As with wheat leaf extracts and horseradish peroxidase (Maclachlan and Waygood, 18, Waygood et al., 24) hydroquinone and its oxidation product p-quinone retarded the catalase-DCP system by 50 per cent at concentrations of 1.1 to 1.3×10^{-5} M. Light had no effect.

Riboflavin and riboflavinphosphate also retarded IAA oxidation in catalase-DCP systems and riboflavin at a concentration of 5×10^{-4} M completely inhibited the reaction in darkness (Figure 2 E). At 2×10^{-5} M it was without effect in light or darkness, but at the intermediate concentrations the retardation which occurred in darkness was strikingly overcome by white or blue light, but not by red light (Figure 2). This is interesting since light had virtually

no effect on IAA oxidation in the absence of riboflavin (Figure 1) and in the presence of riboflavin when catechol was present (Waygood *et al.*, 24). When systems containing riboflavinphosphate were illuminated the amount of oxygen consumed generally exceeded that expected for the theoretical equivalence of IAA. This additional oxygen uptake was apparently due to reversible cofactor oxidation which is promoted by riboflavin, manganese and light (Maclachlan and Waygood, 17, 18).

The rate of IAA oxidation under illumination in the presence of riboflavinphosphate was no greater than the rate in darkness in the absence of riboflavin (Figure 2). The intensity of white or blue light used in these experiments i.e. white: 1380 ft. candles, blue: 220 ft. candles, was well above that required for light saturation. It should be emphasised that light does not activate nor sensitise IAA oxidation, but rather it overcomes the protective effect that riboflavin confers on IAA. The mechanism of riboflavin retardation and the nature of the light effect will be discussed in detail in a later paper (Maclachlan and Waygood, 18) which describes similar effects with the wheat leaf system.

Discussion

Reports of the effect of catalase on the course of the oxidation of IAA have varied widely depending on the source of the system used to catalyse the reaction. Thus catalase completely inhibited IAA oxidation catalysed by pea epicotyl extracts in the dark (Goldacre, 7), extended the lag period with wheat leaf extracts (Waygood *et al.*, 23), enhanced the rate of IAA oxidation catalysed by horseradish peroxidase (Kenten, 13) and had no effect on the non-enzymic breakdown by manganic ions (Maclachlan and Waygood, 16). Evidently. either the catalase extracts or the reactions undergone by IAA were different in each case.

Lack of agreement on the catalase effect and also on the pH optimum, cofactor, manganese and light requirements has led to the supposition that IAA is degraded by different pathways and enzymes in plant tissues (Gortner and Kent, 10). Such a conclusion may appear to be warranted by the discrepancies of the *in vitro* systems, but from a physiological viewpoint metabolic patterns are conveniently similar in different species and indeed in kingdoms. If then IAA catabolism is an important factor controlling auxin level and subsequent growth responses of higher plants it is probable that the metabolic pathway and the enzymes responsible are common to them all. However, before this probability receives experimental justification the conflicting results of the *in vitro* studies must be reconciled.

The present results show that catalase, like peroxidase, catalyses the complete oxidation of IAA provided manganese and an appropriate cofactor are present. Earlier demonstrations that catalase inhibited the IAA 'oxidase' from pea epicotyls (Goldacre, 7) may possibly be explained by the presence in catalase preparations of a powerful, but easily destroyed inhibitor. We have shown that such an inhibitor can be destroyed by light. Catalase-inhibition of pea "oxidase" was also overcome by light (Galston et al., 6) and showed some degree of activation. The activation in light would be caused by the peroxidatic effect of the uninhibited catalase.

It is interesting that preparations of 2,4-dichlorophenoxyacetic acid diminished the inhibition by catalase (Goldacre, 7), but these were later shown to contain some dichlorophenol (Goldacre and Galston, 8). It is generally agreed that dichlorophenol promotes IAA oxidation and although it appears to inhibit the catalatic action of catalase (Goldacre and Galston, 8) its function here is undoubtedly as a redox catalyst since it can be replaced by classical peroxidase substrates e.g. resorcinol, which are active as oxidants of manganese in the Kenten-Mann system (Kenten and Mann, 14).

With regard to reports of IAA "oxidase" systems which show no apparent phenol (Goldacre, 7, Tang and Bonner, 20, Wagenknecht and Burris, 22) or manganese requirement (Goldacre et al., 9, Kenten, 13, Tang and Bonner, 20), it is possible that the extracts used contained these cofactors endogenously. The present authors have found that even after prolonged dialysis of wheat leaf extracts and horseradish peroxidase (Maclachlan and Waygood, 17), some residual activity towards IAA persisted in the absence of either cofactor. It is interesting that this does not apply to catalase. Peroxidase appears to possess an affinity for both manganese and naturally occurring cofactors of IAA oxidation and this would serve to explain previous reports of IAA oxidation in the absence of added cofactors.

Because of these and other considerations discussed elsewhere (Waygood et al., 24) it is suggested that IAA degradation in most of the systems studied in vitro proceeded via the same metabolic pathway and utilised peroxidase or catalase, organic redox catalysts and manganic ions. The question remains as to whether this system operates in the intact plant. At present the evidence in favour of this is mostly indirect, but firstly it is evident that the experimental environment of the system could exist in vivo. Peroxidase and catalase are ubiquitously distributed in the plant kingdom and the former appears to have an affinity for the natural cofactors of IAA oxidation. A natural organic cofactor or mediator of the oxidation occurs at least in wheat leaves and probably elsewhere (Goldacre and Galston, 8, Kenten, 13). Most probably these factors have a monohydric phenolic group and it would not be surprising if they varied from plant to plant and set the

pattern of the growth response to some auxins. Secondly maleic hydrazide, which promotes IAA oxidation by acting as a redox cofactor, is also a widely used growth inhibitor (Leopold and Klein, 15) by virtue of the fact that it shows no optimum at which it is most effective (Maclachlan and Waygood, 17). Galston *et al.* (9) have shown that DCP inhibited the growth of pea stem sections and showed a distinct optimum *in vivo* as it does for IAA oxidation *in vitro* (Maclachlan and Waygood, 17).

As pointed out previously (Maclachlan and Waygood, 16, Waygood *et al.*, 24) there is no doubt that plants can oxidise manganese. Kenten and Mann (14) have shown that manganic ions are generated by any peroxide-producing system coupled to phenol-peroxidase and presumably manganese is oxidised by this mechanism *in vivo*. Thus manganic ions required for the initiation of IAA destruction *in vivo* may be derived autocatalytically as they are *in vitro* or this source may be supplemented by other peroxigenic systems. The contribution of the latter would depend on the juxtaposition of cofactors and enzymes at the site of IAA activity. If peroxidase is distributed throughout the cytoplasm of plant cells whereas catalase is confined to the particulate fractions (Goldacre and Galston, 8, Jagendorf and Wildman, 11), it is possible that both enzymes catalyse IAA oxidation in different parts of the cell.

It has been shown (Andreae, 1) that light-activated riboflavin catalyses the oxidation of manganese via monohydric phenols under certain conditions *in vitro* and there is also some evidence that light promotes the oxidation of manganese in the plant (Arens, 2). The effect of riboflavin on the inactivation of IAA is of particular physiological interest since the action spectrum of phototropic responses and the absorption spectrum of riboflavin are closely related (Galston, 4). Furthermore Galston (4) showed that IAA oxidation catalysed by extracts from pea epicotyls was stimulated by the same region of the visible spectrum which is absorbed by riboflavin. However added riboflavin was not reported to stimulate IAA oxidation in this system. Although Galston *et al.* (5, 6) postulated the participation of a flavoprotein in the reaction sequence, the mechanism of the light effect was not clear especially since the experiments of Kenten (13) rendered improbable the requirement for a flavoprotein component.

On the basis of the present results it appears that riboflavin neither activates nor sensitises IAA oxidation, but on the contrary protects the hormone in darkness and inhibits its breakdown. The inhibition by riboflavin is photoreversible and light has no effect other than to allow normal dark oxidation to take place. It is possible that naturally occurring riboflavin in Galston's system (5, 6) also retarded the oxidation of IAA and light produced its effect by overcoming the inhibition. The parallel which exists

between the effect of light on growth and on the destruction of IAA *in vitro* supports the hypothesis that catabolism of IAA in both follows the same pathway. Thus riboflavin may continuously exert a check on IAA destruction *in vivo*, the extent of which is determined by illumination.

Summary

Catalase mediates the decarboxylation and oxidation of indoleacetic acid by acting peroxidatically in a reaction sequence which involves:

(a) initiation by manganic ions,
(b) peroxide formation,
(c) catalysed peroxidation of organic hydrogen donors,
(d) manganese oxidation.

The peroxidatic activity of catalase is inhibited during the oxidation but the catalatic activity remains unimpared.

Light has no effect on the oxidation of indoleacetic acid by catalase systems unless certain inhibitors are present. Undialysed catalase preparations from beef liver contain a photolabile thermostable inhibitor. Riboflavinphosphate retards IAA oxidation and light of the blue region overcomes its effect. Light and riboflavinphosphate together do not augment the rate of IAA oxidation beyond that in darkness in the absence of riboflavin.

Evidence in favour of the operation of this system in the intact plant is summarized. It is suggested that riboflavin promotes growth by inhibiting indoleacetic acid destruction. Light overcomes the protective effect of riboflavin phosphate *in vitro* and may thereby inhibit growth.

References

1. Andreae, W. A.: The photoinduced oxidation of manganous ions. — Arch. Biochem. and Biophys. 55: 584. 1955.
2. Arens, K.: Physiologisch polarisierter Massenaustausch und Photosynthese bei submersen Wasserpflanzen. — Planta 20: 621. 1933.
3. Chance, B.: The iron-containing enzymes. C. The enzyme substrate compounds and mechanism of action of the hydroperoxidases. — The Enzymes. Sumner and Myrbäck. Ed. 2: 1: 428. Academic Press Inc., New York. 1951.
4. Galston, A. W.: Riboflavin, light, and the growth of plants. — Science 111: 619. 1950.

5. — & Baker, R. S.: Studies on the physiology of light action. III. Light activation of a flavoprotein enzyme by reversal of a naturally occurring inhibition. — Am J. Botany 38: 190. 1951.

6. — Bonner, J. & Baker, R. S.: Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. — Arch. Biochem. and Biophys. 42: 456. 1953.

7. Goldacre, P. L.: Hydrogen peroxide in the enzymatic oxidation of heteroauxin. — Aust. J. Sci. Res. B. 4: 293. 1951.

8. — & Galston, A. W.: The specific inhibition of catalase by substituted phenols. — Arch. Biochem. and Biophys. 43: 169. 1953.

9. — — & Weintraub, R. L.: The effect of substituted phenols on the activity of the indoleacetic acid oxidase of peas. — Arch. Biochem. and Biophys. 43: 358. 1953.

10. Gortner, W. A. & Kent, M.: Indoleacetic acid oxidase and an inhibitor in pineapple tissue. — J. Biol. Chem. 204: 593. 1953.

11. Jagendorf, A. T. & Wildman, S. G.: The proteins of green leaves. VI. Centrifuged fractionation of tobacco leaf homogenates and some properties of isolated chloroplasts. — Plant Physiol. 29: 270. 1954.

12. Keilin, D. & Hartree, E. F.: Catalase, peroxidase and metmyoglobin as catalysts of coupled peroxidatic reactions. — Biochem. J. 60: 310. 1955.

13. Kenten, R. H.: The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems. — Biochem. J. 59: 110. 1955.

14. — & Mann, P. J. G.: The oxidation of manganese by peroxidase systems. — Biochem. J. 46: 67. 1950.

15. Leopold, A. C. & Klein, W. H.: Maleic hydrazide as an antiauxin in plants. — Science 114: 9. 1951.

16. Maclachlan, G. A. & Waygood, E. R.: Catalysis of indoleacetic acid oxidation by manganic ions. — Physiol. Plant. 9: 321. 1956.

17. — — Kinetics of the enzymically catalysed oxidation of indoleacetic acid. — Can. J. Biochem. and Physiol. Nov. 1956.

18. — — Inhibition and retardation of the enzymically catalysed oxidation of indoleacetic acid. — Arch. Bioch. and Biophys. In press.

19. Ray, P. M. & Thimann, K. V.: Steps in the oxidation of indoleacetic acid. — Science 122: 187. 1955.

20. Tang, Y. W. & Bonner, J.: The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedling. — Arch. Biochem. 13: 11. 1947.

21. Tauber, H.: Crystalline catalase. a peroxidase. — Proc. Soc. Exp. Biol. and Med. 81: 237. 1952.

22. Wagneknecht, A. C. & Burris, R. H.: Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. — Arch. Biochem. 25: 30. 1950.

23. Waygood, E. R., Oaks, A. & Maclachlan, G. A.: On the mechanism of indoleacetic acid oxidation by wheat leaf enzymes. — Can. J. Botany 34: 54. 1956.

24. — — — The enzymically catalysed oxidation of indoleacetic acid. — Can. J. Botany. Nov. 1956.

The Uptake of Iron as Ferric Sequestrene by *Vicia faba* and *Phaseolus vulgaris*

By

SAMULI HEINONEN and HARRY WARIS

Botanical Institute, University of Helsinki
(Received September 3, 1956)

In supplying plants with iron, excellent results have been obtained with the chelate compound of ethylene diamine tetraacetic acid or "sequestrene" (Hutner *et al.* 1950, Jacobson 1951, Reischer 1951, Hutner 1953, Hutner *et al.* 1953, Provasoli and Pintner 1953, Burlew 1953, Waris 1953, Krauss 1954). In the presence of EDTA, the iron will not readily precipitate in the nutrient solution nor be bound in the roots in the form of insoluble phosphates, at least not to such an extent as to cause chlorosis and inhibition of growth (De Kock and Strmecki 1954, De Kock 1955). Although it was to be expected that the chelate compound would pass into the ascending sap, a direct analysis of this seemed desirable to elucidate the mechanism of the root's function, all the more so as various chelating agents other than EDTA have been supposed no to enter the root cells (Heck and Bailey 1950).

Methods

The plants were grown in both water and sand cultures. Most of the experiments were carried out with plants grown in water culture, the nutrient solution being aerated. Some of the plants were grown in quartz sand watered with a nutrient solution. In the latter case the supply of nutrients must obviously be more irregular.

At the age of 1—1 1/2 months, the plants were transferred to the final test solutions, their stems were cut off at a height of 1—2 cm. and replaced by graduated pipettes which were connected with the stumps with rubber tubes 1 1/2—2 cm. long, wax or paraffin being used to tighten the joints. The exudate collected in the

pipettes during 24 hours was then subjected to a colorimetric analysis with a Beckman spectrophotometer. The samples analysed measured from 0.1 to 0.65 ml.

The iron was determined as ferric thiocyanate, and the organic radical of ferric sequestrene indirectly from the amounts of ammonium ferric sulphate consumed at titration in the presence of potassium thiocyanate as indicator. This indirect determination of EDTA is subject to considerable errors, as the exudate from plants not supplied with EDTA also contained substances consuming ammonium ferric sulphate at titration. To reduce this error source, various components of the complete nutrient solution were omitted from the final test solutions.

Calibration curves were made from determinations with 0.0005 M ammonium ferric sulphate and 0.001 M ferric sequestrene solutions. On titrating EDTA, 0.5 ml. of the ammonium ferric sulphate solution was found to correspond to 1 milli-mole of EDTA per litre. The amount of ferric thiocyanate was calculated from the light absorption at the wave length 525 mμ. The minimum amounts detectable were considered to be 0.0077 milli-moles per litre for iron and 0.0154 milli-moles per litre for EDTA. The means and standard errors have been calculated, assuming that the minimum amount is present when it is not exceeded, and in some cases, in addition, taking zero for the minimum.

It may be pointed out that in some cases the addition of hydrochloric acid to the exudate, in order to release bound iron, caused a precipitation which evidently led to erroneous results. Such samples, therefore, were omitted from the calculations.

Experiments

The experiments were carried out in two series, the principal difference being in the time the test solutions were allowed to stand for precipitation of the inorganic iron before the plants were transferred to them; for the first series the plants were grown in water culture, and the inorganic iron had had time to precipitate for 24 hours, whereas for the second series the plants were grown in sand culture, and were transferred to the test solutions immediately after the iron source had been dissolved. In fact, the second series was only found to be necessary after the experiments of the first series had been finished. It cannot be decided whether the mode of culture, in water or sand prior to the final tests, affected the results.

The number of replicates is given in brackets after the plant names.

Series I. Plants grown in water culture transferred to the test solutions after precipitation of the inorganic iron.

The complete nutrient solution contained in 100 litres: 100 g KNO_3, 20 g. $(NH_4)_2HPO_4$, 20 g. $MgSO_4 \cdot 7H_2O$, 33 g. $CaCl_2$ (fusum), 2 g. $MnSO_4 \cdot 4H_2O$, 0.25 g. $FeSO_4 \cdot 7H_2O$, 0.524 g. of the disodium compound of EDTA (Komplexon III from B. Siegfried AG), 5.68 g. $KHCO_3$, 0.2 g. $ZnSO_4 \cdot 7H_2O$, 0.15 g. ammonium molybdate, 0.05 g. $CuSO_4 \cdot 5H_2O$, 0.05 g. $CoSO_4 \cdot 7H_2O$, and 0.05 g. KI; pH adjusted to about 6. The final test solutions were made up of

a standard solution to which the substance to be tested was added. The standard solution contained in 100 litres: 20 g. KNO_3, 2 g. $MnSO_4 \cdot 4H_2O$, and additional trace elements as in the complete nutrient solution; pH about 6. After the addition of iron, the test solution was allowed to stand for 24 hours.

A. Test solution with 0.43 g. or about 0.9 milli-moles of ammonium ferric sulphate per litre added, resulting, after precipitation, decanting and filtering, in a solution of ferric hydroxide and ferric oxide hydrate with about 0.021 milli-moles of iron per litre. The iron content of the exudate varied in Vicia (12 replicates) from the minimum detectable up to 0.26 milli-moles per litre, in Phaseolus (9), up to 0.25 milli-moles, the respective means being for Vicia 0.032 ± 0.010 (or if zero is taken for the minimum, 0.027 ± 0.009), for Phaseolus 0.034 ± 0.009. Thus, in both cases, a little more was found in the exudate than in the test solution, the difference being, however, insignificant.

B. Test solution with the ferric sodium compound of EDTA (ferric sequestrene).

1) 6.81 milli-moles added per litre. The iron content of the exudate in Vicia (23) 0.10—1.50, mean 0.401 ± 0.082; in Phaseolus (1) 0.48 milli-moles per litre.

2) 13.62 milli-moles added per litre. Iron in the exudate: Vicia (5) 0.07—0.70, mean 0.318 ± 0.084; Phaseolus (7) 0.09—0.50, mean 0.254 ± 0.069.

C. Test solution with the disodium compound of EDTA (Komplexon III). "EDTA" denotes substances consuming ammonium ferric sulphate on titration, as does EDTA.

1) 1.409 milli-moles added per litre. "EDTA" in the exudate of Vicia (8) 0.393 ± 0.049, in that of Phaseolus (6) 0.404 ± 0.041 milli-moles per litre.

2) 7.04 milli-moles added per litre. "EDTA" in Vicia (17) 0.735 ± 0.104, in Phaseolus (3) on an average 0.435 milli-moles per litre.

D. Controls for the indirect calculations of the EDTA content in the exudate of Vicia. The number of replicates in brackets.

1) Standard solution without additions. "EDTA" (5) 0.143 ± 0.072 milli-moles per litre.

2) Test solution A. "EDTA" (4) 0.320 ± 0.195 milli-moles per litre.

3) Test solution B 1. "EDTA" (5) 0.278 ± 0.010 milli-moles per litre.

Calculations from the above data for Vicia:

$$\text{B1—A:} \quad 0.401\,(\pm 0.082)—0.032\,(\pm 0.010) = 0.369 \pm 0.088$$
$$\text{B2—B1:} \quad \text{No significant difference}$$
$$\text{C1—D1:} \quad 0.393\,(\pm 0.049)—0.143\,(\pm 0.072) = 0.250 \pm 0.087$$
$$\text{C1—D2:} \quad \text{No significant difference.}$$
$$\text{C1—D3:} \quad 0.393\,(\pm 0.049)—0.278\,(\pm 0.010) = 0.115 \pm 0.042$$

C2—C1: 0.735($\pm$0.104)—0.393($\pm$0.049)=0.342$\pm$0.115
C2—D1: 0.735($\pm$0.104)—0.143($\pm$0.072)=0.592$\pm$0.126
C2—D2: 0.735($\pm$0.104)—0.320($\pm$0.195)=0.415$\pm$0.221
C2—D3: 0.735($\pm$0.104)—0.278($\pm$0.010)=0.457$\pm$0.109

Series II. Plants grown in quartz sand watered with a nutrient solution were transferred to the test solutions immediately after their preparation. The substances to be tested were dissolved in distilled water, the pH being adjusted with sodium hydroxide and hydrochloric acid.

A. 50 mg. of $FeSO_4 \cdot 7H_2O$ added per litre, corresponding to about 10 mg. or 0.18 milli-moles of Fe.

1) pH at the beginning 6.0, at the end 5.3. Iron at the end in the solution 0.095 milli-moles per litre. Iron in the exudate of Phaseolus (5) 0.015—0.038, mean 0.021; in Vicia (2) 0.066 and 0.43 (?) milli-moles per litre.

2) pH at the beginning 3.5, at the end 3.7. Iron in the solution at the end 0.112 milli-moles per litre; in the exudate of Phaseolus (3) 0.013—0.018, mean 0.015; in Vicia (2) 0.076 and 0.091 milli-moles per litre.

B. Test solution otherwise as in A, but with an addition of EDTA (Komplexon III) equimolar to the iron salt.

1) pH at the beginning 6.0, at the end 4.5. Iron in the solution at the end 0.106 milli-moles per litre. Iron in Phaseolus (4) 0.013—0.038, mean 0.020; in Vicia (2) 0.018 and 0.034 milli-moles per litre.

2) pH at the beginning 3.5, at the end 3.6. Iron in the solution at the end 0.116 milli-moles per litre; in the exudate of Phaseolus (4) 0.015—0.062, mean 0.036; in Vicia (1) 0.042.

Conclusions

A survey of the data presented in the above experiments shows that the number of parallels is not in all cases great enough to allow an exact statistical analysis, but it is sufficient for certain conclusions about the effect of EDTA on the uptake of iron.

From the first series of experiments (I), the following conclusions can be drawn:

1. From the inorganic solution used, at pH 6, iron had been taken up in about the same concentration as it was present in the solution, to judge from its concentration in the exudate. One should note that in the experiments concerned the concentration of iron in the solution, after precipitation had taken place, was very low.

2. Considerably more iron could be supplied in the chelate form than in

the inorganic form, and consequently more iron was found in the exudate, the difference being roughly tenfold. It seems that a certain maximum content of the chelate iron in the exudate could not be exceeded, as the higher of the two concentrations applied did not result in any higher content in the exudate (cf. Calculations, B2—B1).

3. By the method applied, only some indirect evidence could be adduced in favour of the view that the chelate compound of iron may pass as such from the nutrient solution into the ascending sap. This evidence consists of the fact that in the exudate the amount of substances consuming ammonium ferric sulphate at titration, as does EDTA, could be increased by increasing the concentration of the disodium compound of EDTA in the nutrient solution (cf. Calculations, C2—C1, for Vicia). However, the indirect calculation of the EDTA content in the exudate seems to be subject to rather large errors apparently due to substances other than EDTA likewise consuming ammonium ferric sulphate at titration.

The second series of experiments (II) was carried out later than the first in order to find out whether by the addition of EDTA to the nutrient solution an increase in the iron content of the exudate could be brought about even as compared with an inorganic solution high enough in iron. In these experiments, the iron content found in the exudate exhibited great individual variations which may partly, but not wholly, be due to experimental errors. (The value 0.43 for Vicia in the experiment A1 seems to be either exceptional or erroneous).

Although the parallel experiments in series II are few in number, it appears that in the presence of EDTA, both at the higher and the lower initial pH values, the iron content of the exudate might be higher or lower than when the iron was present in inorganic form only.

Summary

The ability of *Vicia faba* and *Phaseolus vulgaris* to take up iron from solutions with and without EDTA was investigated by analysing the exudates which were collected in pipettes after the stems had been cut off.

The results indicate that the higher iron content of the exudate in the presence of the chelate compound as compared with an inorganic iron source is mainly referable to the fact that the inorganic iron tends to be removed from the nutrient solution by precipitation, whereas, within the error limits, no considerable differences was found between the two iron sources when their concentrations in the solutions were high enough, the individual variations being in both cases rather large.

There was some indirect evidence in favour of the view that the iron may pass in the chelate form from the nutrient solution into the ascending sap.

The authors are indebted to Mr. Osmo Mäkitie, M.A., for the analyses of series II.

References

Burlew, J. S.: Algal culture from laboratory to pilot plant. — Carnegie Institution of Washington Publication 600. Washington 1953.

De Kock, P. C.: Influence of humic acid on plant growth. — Science 121: 473. 1955.

— & Strmecki, E. L.: An investigation into the growth promoting effects of a lignite. — Physiol. Plant. 7: 503. 1954.

Heck, W. W. & Bailey, L. F.: Chelation of trace metals in nutrient solutions. — Plant Physiol. 25: 573. 1950.

Hutner, S. H.: Comparative physiology of heterotrophic growth in plants. — Loomis, W. E.: Growth and Differentiation in Plants. Ames 1953.

— Provasoli, L., Schatz, A. & Haskins, C. P.: Some approaches to the study of the role of metals in the metabolism of microorganisms. — Proc. Amer. Phil. Soc. 94: 152. 1950.

— Provasoli, L. & Filfus, J.: Nutrition of some phagotrophic freshwater chrysomonads. — Ann. N. Y. Acad. Sci. 56: 852. 1953.

Jacobson, L.: Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. — Plant Physiol. 26: 411. 1951.

Krauss, R. W.: Absorption of inorganic nutrients by Scenedesmus. — Carnegie Institution of Washington Year Book 53: 179. 1954.

Provasoli, L. & Pintner, I. J.: Ecological implications of in vitro nutritional requirements of algal flagellates. — Ann. N. Y. Acad. Sci. 56: 839. 1953.

Reischer, H. S.: Growth of Saprolegniaceae in synthetic media. I. Inorganic nutrition. — Mycologia 43: 142. 1951.

Waris, H.: The significance for algae of chelating substances in the nutrient solution. — Physiol. Plant. 6: 538. 1953.

Growth and Metabolism of *Ophiostoma multiannulatum* on Different Sources of Carbon

By

Bengt V. Hofsten

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden
(Received September 11, 1956)

Introduction

The use of the ascomycete *Ophiostoma multiannulatum* in the field of biochemical genetics was suggested by Fries (1), who has obtained a great number of mutant strains showing varying degrees of heterotrophy. In contrast to the much used fungus Neurospora, most Ophiostoma strains can grow in the form of budding conidia in liquid media. This suggested attempts to use a continuous culture technique for the study of genetical and physiological properties of some strains. A slight modification of the apparatus developed by Monod (2) was found useful for Ophiostoma, and cultures have been kept growing without contamination in such a device for several weeks (3).

In connection with the development of this technique, a broadened knowledge of the nutrition and metabolism of the fungus seemed desirable. For this work a typical wild strain has been used, but comparative studies with different mutant strains of the organism are being planned. In comparison with the wealth of literature on growth and metabolism of the related Neurospora, the data presented here are of necessity very limited in scope, and the results are therefore presented without any claim of completeness. It is possible to obtain large quantities of physiologically very active cells from simple synthetic media, and it is therefore likely that the organism will provide a suitable experimental material for more detailed metabolic studies as soon as a gentle process of preparing cell homogenates is found.

The use of a continuous culture technique in the study of enzyme synthesis

[624]

was introduced by Monod and Cohn (4), who worked with *E. coli*. Recently A. Davies (5, 6) has applied the technique with great success to the yeast *Saccharomyces fragilis,* and the interest in continuous cultures is rapidly increasing as emphasized in the review by Novick (7). When the method was tried with Ophiostoma, the most immediate problem was to obtain a homogeneous conidial growth. A culture grown on glucose at 30° could be maintained for many days, but growth on the glass wall was often a serious difficulty, because Ophiostoma tends to grow as hyphae on a surface. When other conditions of growth were tried, morphological changes of the cells easily occurred, and the application of continuous cultures for the study of the enzyme synthesis was therefore greatly limited. A study of factors influencing the growth morphology of Ophiostoma is presented elsewhere (8), and only some general experiences from continuous culture experiments are given here. The apparatus used, has been described earlier (3), and only minor technical improvements have been made since then.

Material and Methods

Ophiostoma multiannulatum strain No. 51 (in the following briefly called only Ophiostoma) was maintained by regular transfers on agar slants containing: malt extract (Difco) 25 g, yeast extract (Difco) 0.5 g, yeast nucleic acid, hydrolyzed with ammonia, 1 g, agar 15 g and water 1000 ml.

Growth in liquid cultures was started by transferring a small piece of agar-growth to 100 ml Pyrex flasks containing 20 ml of a solution prepared according to the following description:

Solution A:			
NH_4-tartrate	5 g		
L-Asparagine	1 g		
KH_2PO_4	1 g		
$MgSO_4 \cdot 7H_2O$	0.5 g		
NaCl	0.1 g		
$CaCl_2$	0.1 g		
$ZnSO_4 \cdot 7H_2O$	4.4 mg		
$MnSO_4 \cdot 4H_2O$	4.1 mg		
Fe-citrate	4.5 mg		
Citric acid	4.5 mg		

Inositol	10 mg
Thiamin	0.1 mg
Pyridoxin	0.1 mg
Dist. water	900 ml

Solution B:

Carbon source	5 g or less
(Glucose if not otherwise stated)	
Dist. water	100 ml

Each flask was provided with 18 ml, of solution A and then autoclaved at 120° for ten minutes. Solution B was autoclaved separately or, when instead of glucose, heat-sensitive carbohydrates were used, filtered sterile through Pyrex filters. Two ml of solution B was then added to solution A under aseptic conditions. If the two solutions were autoclaved together, this resulted in yellowing of the solution and a distinct decrease in the growth rate of the organism. The vitamins were supplied in the form of a solution which could not be stored for more than a short time. Otherwise the

growth rate was considerably reduced, a fact which initially caused much trouble in continuous culture experiments.

The flasks were incubated in constant-temperature rooms on reciprocal shakers making two doublestrokes per second. Growth experiments were also performed in 5 liter Roux bottles and 16 mm Pyrex tubes incubated on shaking machines.

Growth of the fungus was estimated by measuring the increase in turbidity determined at 600 mμ with the aid of a Beckman B spectrophotometer. Extinction figures could be converted to dry weight values with the aid of calibration curves, which were checked regularly. When samples from a culture gave extinction values over 1, they were diluted so that usually only the almost linear part of the calibration curve was used.

In some experiments, growth was followed directly in shaking tubes containing 10 ml of solution. A combination of flask and tube suggested by Ballentine (9) was also tried with rather good results.

The β-glucosidase and β-galactosidase activities were determined by slight modifications of the procedures outlined by Hestrin et al. in Methods in Enzymology (10). The chromogenic substrates ortho-nitrophenyl-β-galactoside (ONPGal) and para-nitrophenyl-β-glucoside (PNPGluc) were used at a concentration of 0.001 M, and the hydrolysis of them was followed in Na-Acetate buffer at pH 5 under zero-order conditions.

Respiration of the cells was measured by conventional Warburg technique according to Umbreit et al. (11). CO_2-uptake was followed by the direct method, and the endogenouos respiration of a cell preparation was always checked during the entire experiment. Each vessel with a total volume of approximately 15 ml contained as a rule 1.8 ml of a suspension of washed cells in 0.056 M phosphate buffer pH 6.0. If necessary, the substrates were adjusted to pH 6.0, and the pH of the contents of the Warburg vessels were checked after the experiments.

Experimental

When Ophiostoma is grown in a synthetic medium under conditions which favour conidial growth, the cells, which have a density of slightly above 1, contain 80—85 per cent water. The nitrogen content of the cells varies with the nutritional conditions, but is between 8.5—9 per cent of dry the weight, if they are grown in media with an easily assimilable nitrogen source. When hyphal growth is favoured, as in the presence of malt extract, the nitrogen content of a mature culture is about 6.5 per cent, probably because of autolysis in parts of the hyphae. If cells are grown in a nitrogen deficient medium, the nitrogen content goes down to about 5 per cent within ten hours, at the same time as typical changes in the morphology occurs.

Qualitative tests have shown that the cell wall contains a high quantity of chitin, and this makes them so resistent that they withstand several hours of boiling in 6 M HCl or 40 per cent KOH. Several different methods of disrupting the fresh cells have been tried without success, and this has reduced the possibilities of making experiments with cell homogenates. Lyophilized cells

are however relatively easily broken in a Potter-Elvehjem homogenizer or when ground with glass powder.

Ophiostoma is completely dependent on aerobic conditions for growth, and the cells die rapidly in the absence of air if they are not kept in the refrigerator. Efficient aeration of all cultures is therefore necessary, and experiments with continuous cultures have shown that maximal growth rate is only obtained with high aeration rates.

When centrifuged Ophiostoma cells have been kept in the refrigerator for some time, they become weakly pink. If they are examined through a pocket spectroscope they show a strong absorption band at 550 mμ and possibly some weak absorption between 520 and 530 mμ. When such cells are aerated the absorption largely disappears, but can be restored by the addition of sodium hydrosulfite. A differential spectrum obtained between reduced and oxidized cells and measured in a Beckman DU Spectrophotometer shows a distinct peak at 550 mμ, whereas the absorption at other wavelengths is less marked. This shows that cytochrom c is a main component of the respiratory system of Ophiostoma.

The morphology of Ophiostoma is highly dependent on the temperature of incubation, but growth is obtained at all temperatures from 4° to 30°, with most rapid growth between 25° and 30°. Higher temperatures are inhibitory for cell division, and very poor growth is obtained over 31°.

The pH dependence of Ophiostoma is not particularly high, and growth is obtained from pH 3.5 to 7 with most rapid growth about 5.5. Some acid is produced during the growth on 0.5 per cent sugar or glycerol, but the tartrate and phosphate of the normal synthetic medium prevents large pH-fluctuations. When the glucose concentration is lowered, a rise in pH is obtained, probably because asparagine is partly used as a carbon source which results in liberation of excess ammonia. If asparagine is omitted from the medium, growth is less rapid, and attempts to provide the necessary nitrogen in the form of inorganic ammonium salts, have given very poor growth and lowered pH.

Growth on glucose

Figure 1 illustrates the growth of Ophiostoma on 0.5 per cent glucose at 30° in a Roux bottle. The length of the lag phase depends on the physiological state of the inoculated cells, and may vary from zero to about twelve hours. During this time all cells elongate very much before conidia are being liberated. The conidia are then multiplying by a budding process, which for most cells implies a division into two almost identical cells.

During growth metabolic products are excreted into the medium, and with a continuous culture composed of only living cells, culture filtrates have been
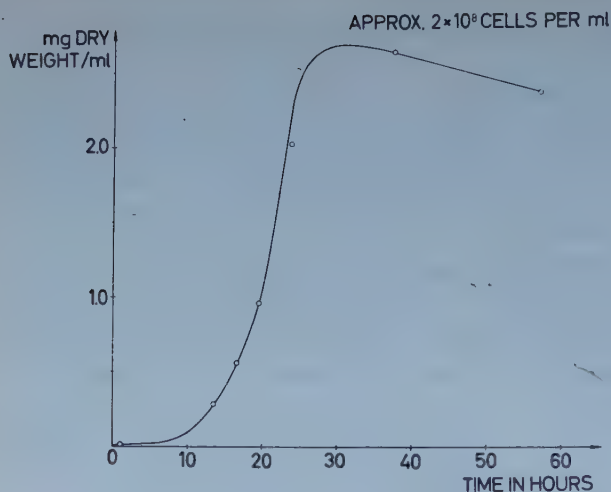
Figure 1. *Growth of Ophiostoma in a synthetic medium with 0.5 per cent glucose.*

shown to contain at least ten amino acids together with other unidentified ninhydrin positive substances and some protein material. This condition seems to be of quite general occurrence among microorganisms, and Morton and Broadbent (12) have recently studied this phenomenon in some fungi.

Growth on other monosaccharides

Both fructose and mannose are easily assimilated by Ophiostoma whereas galactose gives no growth. Of other compounds tried, rhamnose and xylose give some growth but fucose, arabinose, sorbose and ribose are unavailable
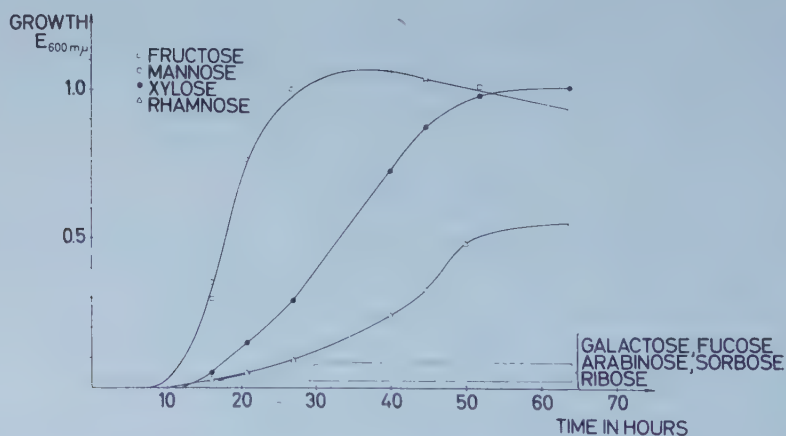
Figure 2. *Growth of Ophiostoma in shaking tubes on various monosaccharides.* The extinction values are not directly proportional to the amount of growth.

under the given growth conditions. Figure 2 illustrates growth curves obtained with shaking tubes containing 0.5 per cent of the carbon source in the normal synthetic medium.

Growth on oligo- and polysaccharides

The wild strain of Ophiostoma can grow without lag and with normal growth rate on maltose and sacchrose. Cellobiose, lactose, trehalose and raffinose are less easily utilized and with a more or less marked lag. Melibiose, melizitose cellulose, starch and glycogen are not attacked to any apparent extent, and tests for cellulolytic activity in cultures have been negative.

Production of β-glucosidase and β-galactosidase

The synthesis of β-glucosidase and β-galactosidase by Ophiostoma has been studied in some detail, and preliminary data on the latter enzyme have been published (13). Figure 3 illustrates the growth on lactose and cellobiose in media with and without the addition of casein hydrolyzate. The result suggested that the involved enzymes were inducible, and that the synthesis of them was facilitated by an external supply of amino acids. However, a culture grown on glycerol was tested for activity towards the substrates ONPGal and PNPGluc, enzyme activity was found both in the cells and in the cell-free medium. The presence of lactose or cellobiose in various concentrations in the medium did not cause more than a twofold or smaller increase in enzyme activity. Neither galactose nor melibiose gave any significant increase in β-galactosidase activity. If lactose grown cells were inoculated into a new lactose medium, no growth occurred until after a lag period of about the same magnitude as if glucose cells had been used for the inoculation. This can be explained by assuming that the cells are poorly permeable to lactose and cannot grow until they have started to produce β-galactosidase into the medium. As will be seen later, Ophiostoma does not respire lactose easily, and this can be explained by the same assumption. Cellobiose evidently enters the cells more easily, and the adaptive growth on this compound is also pronounced.

Experiments to follow the synthesis of β-glucosidase and β-galactosidase in growing cultures have been complicated by the fact that the enzyme activities are found both in the cells and in the medium. When the cell-free medium and the cells are assayed in parallel, much of the activity is found in the medium. Thus, when samples from a continuous culture on glycerol were incubated with the chromogenic substrates, more than 50 per cent of the total enzyme activity is found in the medium. Toluene treatment of the cells does not give active preparations, and no other way of obtaining the enzymes quantitatively from the cells has yet been found. It is therefore impossible to assert that the activity found in a sample is a true measure of the amount of the enzyme.

Figure 4 illustrates a growth curve on glycerol with the β-glucosidase activities of samples with and without cells presented in the same diagram. Without asparagine in the medium, only about half the enzyme activity is obtained; a result which shows the importance of the nitrogen source for the enzyme synthesis. With glucose as car-
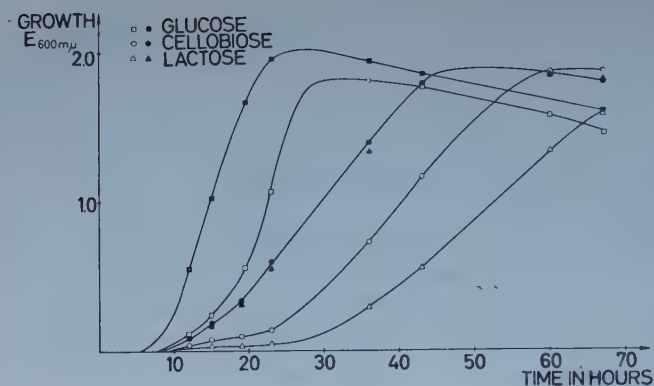
Figure 3. *Growth of Ophiostoma on glucose, cellobiose and lactose.* Open symbols: synthetic medium without asparagine. Solid symbols: acid hydrolyzed casein added to a final concentration of 0.1 per cent.

bon source no enzyme activity is found until practically all glucose has been used up. This is probably a consequence of the now well exemplified inhibiting effect of glucose on the synthesis of this group of enzymes (14).

Some kinetic investigations of the enzymes have been made on partially purified preparations from cell-free media. The amount of protein in such solutions is extremely small, and it proved impossible to use any conventional precipitation procedure even after concentration of the medium. Several adsorption methods were tried, and Dowex 2 in Cl-form was found useful and had the further advantage of being easily regenerated. The application of Dowex 2 for protein chromatography has been studied in detail by Boman and Westlund (15), and in this connection only a brief outline of the procedure used for Ophiostoma media is given.

The cell-free medium was adjusted to pH 7.2 and made 0.02 M with respect to
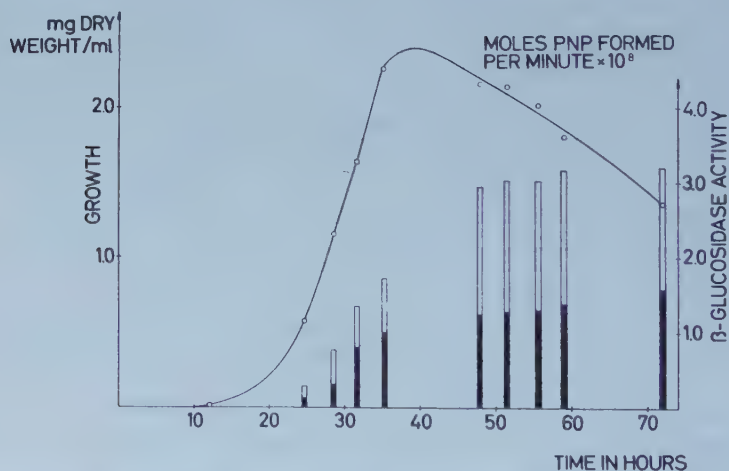
Figure 4. *Growth and production of β-glucosidase in a synthetic medium with 0.5 per cent glycerol.* The right-hand ordinate refers to the bar graph. The total height of the bars indicates the enzyme activity of samples including the cells, whereas the dark portions refers to the activity of the cell-free supernates.

Figure 5. *Activity of β-glucosidase from Ophiostoma at various pH-values.* The enzyme activity is given as the initial velocities, and the stability of the enzyme preparation was checked at the given pH.
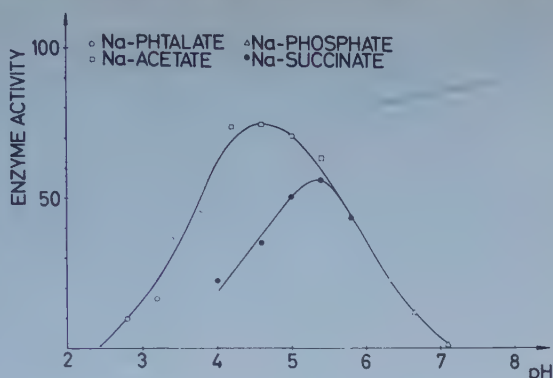
tris-(hydroxy)methyl aminomethanhydrochloride buffer (Sigma Chemical Co., Tech. Bull. 106), pH 7.2. It was then poured on a column of Dowex 2 in Cl-form and in equilibrium with buffer, after which the enzymes could be eluted at a higher chloride concentration, in the presence of the mentioned buffer system, which effectively prevents large pH-changes during the elution. About 80 per cent of the applied enzyme activity could thus be eluted in a small volume at a chloride concentration of 0.6 M with about thirty times the original enzyme activity per ml solution. The increase in specific activity is difficult to give because of the very small amount of protein in the original medium. Cations and neutral compounds pass through the column during the adsorption, whereas anions are held very strongly by the resin. The protein adsorbing capacity of Dowex 2 is rather low, but this is counterbalanced by the efficiency with which impurities can be removed. With Ophiostoma cultures, one volume of resin has completely adsorbed all enzyme activity in six to eight volumes of solution.

Further purification of the enzymes has been achieved by chromatography on calcium phosphate according to Tiselius *et al.* (16), and with the aid of zone electrophoresis, but complete separation of the two activities has not yet been achieved. Preparations which are several thousand times more active than the original medium have thus been obtained, and further chemical characterisation of the two enzymes is being attempted.

Both β-glucosidase and β-galactosidase of Ophiostoma have pH-optima around 5, and they are stable over a wide pH range. Figure 5 illustrates the activity of a β-glucosidase preparation at different pH values. Alberty (17) has discussed the marked influence of different buffer systems on the pH-optima of enzymes, and it is evident that similar conditions apply to the present enzymes.

Several attempts to find out whether the enzymes are specificially dependent on K, Na or any other metal for maximal activity have been made, but the experiments have not given any positive evidence. Ionic strengths higher than 0.02 during the enzyme reaction is attended by a slower reaction rate, but this may be the result of a change in the Michaelis constant of the enzymes.

Growth on other carbon sources

Ethanol and glycerol are easily utilized by Ophiostoma, but both are toxic in higher concentrations. Thus, at more than 0.1 per cent, growth on ethanol
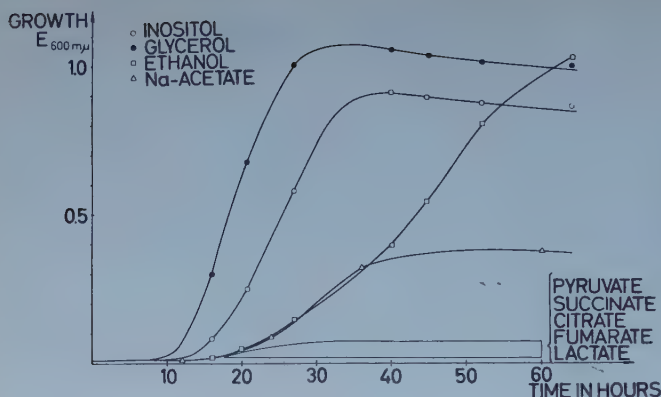
Figure 6. *Growth of Ophiostoma in shaking tubes on various carbon sources other than saccharides.* The extinction values are not directly proportional to the amount of growth.

requires a period of adaptation, whereas lower concentrations give a normal growth response.

Acetate is a very poor carbon source, and the growth is slow and accompanied by morphological changes. Pyruvate, citrate, succinate, fumarate and lactate are not utilized at a concentration of 0.1 per cent or more. The tartrate of the normal synthetic medium is not used as a sole source of carbon, and amino acids, glutamine and asparagine are only assimilated in the presence of a carbon source.

Inositol is normally incorporated in the medium for Ophiostoma, and several mutant strains have an absolute requirement for it. The wild strain does not grow rapidly without an external supply of it, but inositol can also be utilized as a carbon source after a period of adaptation probably involving the induced synthesis of one or several enzymes.

Growth curves obtained on some of the mentioned compounds are illustrated in Figure 6.

Continuous culture experiments

When the growth rate of a glucose-limited culture was varied, morphological changes occurred. The most homogeneous growth was obtained with generation times of 3 to 7 hours, whereas more than ten hours favoured hyphal growth. Nutritional conditions which required comparatively slow growth rates likewise gave rise to a higher proportion of filamentous cells, and nitrogen deficient cultures developed into a tangled mass of hyphae.

In some early experiments, the cultures did not always come into equilibrium at or near 30°, but thinned out more or less rapidly. When the temperature of the actual culture was measured, it was found to be 2—3° higher than the thermostated room in which they were kept. The heat produced during growth may thus make temperature control critical when experiments are

performed near the upper temperature limit of growth of an organism. When the temperature was lowered to less than 25° on the other hand, hyphal growth was favoured, and it became impossible to continue experiments for any length of time.

When growth rate and temperature of incubation was varied within the range which permitted homogeneous growth, no significant difference in enzyme activity of samples from the culture was found. A distinct decrease in the cell production was however noticed when the growth rate was lowered. This observation, which has also been made with bacteria by other authors (18), was however not studied in any detail.

The addition of lactose, galactose or melibiose in various concentrations to a glucose- or glycerol-limited culture, did not cause a marked increase in the β-galactosidase activity. This peculiar result may however depend on the fact that the assay of the enzyme did not give a true estimate of the enzyme production of the cells.

Manometric experiments

Ophiostoma cells show a high endogeneous respiration, which continues for several hours. This indicates that they contain reserve carbohydrates, and tests for glycogen-like material have also been positive. The respiratory activity on glucose is influenced by the age and culture conditions of the cells, and highest Q_{O_2}-values are obtained with cells from a continuous culture. If such cells are washed and shaken with buffer for a short time, they do not give any measurable growth during ordinary Warburg experiments. It is clear that such cells may be physiologically different from cells harvested from the stationary phase of ordinary shaken cultures, but no investigation of this problem has been made.
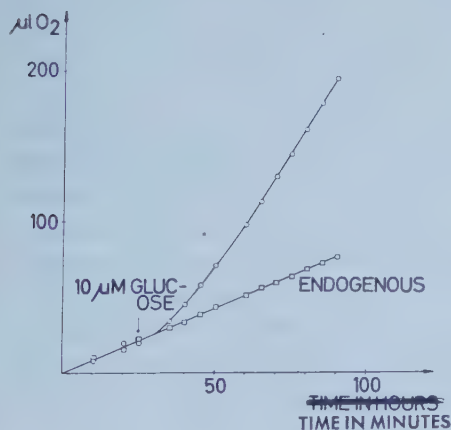
Figure 7. *Respiration of Ophiostoma on glucose.* Two ml of a washed suspension of cells (4.7 mg dry weight) in 0.05 M Na phosphate buffer pH 6.0. Glucose added to one of the flasks after 25 minutes.

Table 1. *Respiration of Ophiostoma on a number of substrates.* Each flask contained two ml of a suspension of glucose-grown cells (4—5 mg dry weight) in 0.05 M Na-phosphate buffer, pH 6.0. The amount of substrate was 10 µMol per flask, and the respiration was followed for several hours to check if a significant change in the respiration occurred.

Substrate	Q_{O^2}, µl O_2 uptake per hr per mg dry weight (Initial, linear rate)	Remarks
None	10—12	Declines to 8—10 after about two hours
Glucose	43—45	Continuously grown cells give more than 50
Glucose-1-phosphate	10	
Gluconolactone	15	
Fructose	37	
Mannose	44	
Galactose	24—26	Almost linear for several hours
Sorbose	29	
Ribose	12	No adaptation noticed
Xylose	20	
Arabinose	11	
Maltose	30	
Saccharose	46	
Lactose	11—15	Lactose-grown cells: 15—20
Melibiose	11	
Cellobiose	24	
Rhamnose	14	
Raffinose	19	
Trehalose	28	
Inositol	17	Inositol-grown cells: 31
Mannitol	20	
Sorbitol	18	
Erythrit	14	
Glycerol	45	
Ethanol	30	High amounts are inhibitory
Acetate	35	
Pyruvate	14	
Lactate	12	
Succinate, citrate	11	
Aspartate, glutamate	12	
Asparagine	15	
NH_4-tartrate	12	

The oxidation of glucose proceeds with a respiratory quotient of approximately 1.0 and is illustrated in Figure 7. The Q_{O_2} on glucose is about 45, proceeding to approximately 30 per cent of the theoretical value for complete oxidation of the substrate. Glycerol is respired as easily, but with a R.Q. of about 0.9. A number of other substances which have been tested as respiratory substrates are listed in Table 1, which gives the Q_{O_2}-values obtained on some of them.

As to the mechanism by which Ophiostoma oxidizes carbohydrates, the experimental data are yet too limited to give any detailed information.

Table 2. *Respiration of Ophiostoma on 10 μMols of glucose in the presence of inhibitors.*

Inhibitor	Concentration of inhibitor during respiration M	Q_{O_2}, μl O_2 uptake per hr per mg dry weight (Initial, linear rate)
No substrate	—	12
No inhibitor	—	44
NaF	0.01	45
»	0.001	45
Iodoacetate	0.001	40 (declining)
»	0.0001	43
2,4-dinitrophenol	0.01	26
»	0.001	55
NaN$_3$	0.001	21 (declining)
»	0.0005	58
»	0.0001	66

Table 2 gives some data on the respiration of glucose in the presence of some inhibitors. The relative insensibility to fluoride and iodoacetate is evident, and growth experiments have shown that the rate of growth of Ophiostoma is only decreased to about 50 per cent in the presence of 0.025 M NaF, whereas 0.012 M fluoride has no influence at all.

Discussion

Most recent investigations concerning the metabolism of carbon compounds by microorganisms have shown that several different metabolic pathways may exist parallelly in the cells. The tricarboxylic acid cycle seems to play an indispensable role in the supply of certain carbon chains for the organism, but its quantitative role as an energy source is a matter of controversy. The unique importance of acetyl coenzyme A in biosynthesis has been well established, but the reactions down to triose and acetyl fragments seem to be manyfold and not always dominated by the Embden-Meyerhof scheme. Different pathways may dominate the metabolism when the cells are actively growing and building up new cell material, and when they are just respiring a given substrate. This complicates the quantitative evaluation of metabolic reactions found in an organism, even when it is strictly aerobic, so that many side reactions may be ruled out.

The mechanisms for the degradation of carbohydrates are extremely variable among micro-organisms, as emphasized in the review by Gunsalus et al. (19). It is very likely that all oligosaccharides are converted by hydrolysis or phosphorolysis to monosaccharides and these in turn to intermediates of glucose or ribose metabolism. The use of whole cells for the investigation of the metabolism of an organism is complicated by permeability problems,

and negative findings do not necessarily mean that the organism does not
metabolize a given compound. In addition, a change in experimental condi-
tions such as temperature of growth and pH may give different results, and
the wellknown adaptive growth response may further complicate the situa-
tion. As now generally accepted, adaptive growth may be caused by several
different mechanisms. Selection plays an important role in the establishment
of clones with changed nutritional behaviour, and genetic changes in a strain
may give rise to adaptation phenomena which are difficult to interpret. The
induced synthesis of enzymes has been shown to be of extreme importance
in the metabolism of all cells. There is every reason to believe that the classi-
fication of enzymes into adaptive and constitutive more is an indication of
quantitative differences than a strict division into groups of different nature.
It has been postulated that most enzymes are synthesized as a response to
inducers, which usually are their natural substrates. Under all circumstances,
the enzyme formation requires not only the presence of an inducer but also
an energy source, the necessary raw material: the amino acids and probably
specific nucleic acids.

A. Davies has published two reports on invertase and lactase formation in
Saccharomyces fragilis (5, 6), which demonstrate how complicated the situa-
tion is in this organism. Not only is the enzyme synthesis inhibited by the
presence of many sugars, but the author found that the lactase activity always
was lower in the intact organism than in the disrupted cells. Such results
must lead to great caution in the interpretation of experiments with whole
cells, and no detailed attempt of explaining the behaviour of Ophiostoma is
therefore made until more experiments can be performed at the enzyme level.
The ability to grow on lactose varies with different strains of the organism,
and some inositol requiring mutants are completely unable to utilize this
saccharide (20). All tested strains grow however on cellobiose, and this com-
pound is also readily used for respiration of the organism. It is not known
whether phosphorolytic cleavage and phosphorylation plays a role in the
assimilation by Ophiostoma, but it is evident that the cells are poorly perme-
able to some compounds which are very probable intermediates in a phos-
phorylated form.

The finding that Ophiostoma secretes β-glucosidase and β-galactosidase out
into the medium has a parallel in the work of Mandels (21), who has studied
invertase formation in *Myrothecium verrucaria*. In his case, 45 per cent of the
total enzyme is found in the cell-free filtrates, and the experiments show
clearly that the enzyme must be released from living spores during germina-
tion. As is also the case with Ophiostoma, no enzyme is liberated from the
cells, if these are incubated with only buffer, and enzyme synthesis thus
seems to be closely related to the growth of the organisms.

In most micro-organisms, where the existence of a direct, oxidation pathway has been looked for, more or less certain positive evidence has been obtained. Although it is likely that some reaction steps of the hexose monophosphate shunt may vary, this pathway is probably indispensable for the metabolism of tetrose and pentose compounds. The data obtained with Ophiostoma is too incomplete to be more than an indication of the existence of the hexose monophosphate shunt, and further work is required to characterize the participating enzymes.

The finding that Ophiostoma can respire on galactose, although this compound is not utilized as a sole source of carbon for growth, requires some consideration. It could be explained by assuming that galactose is inhibitory to cell division, but this is not very likely because the presence of galactose in a glucose medium does not change the growth rate of the organism. An alternative hypothesis would be that galactose can not be transformed into glucose intermediates but is oxidized directly to galactonic acid. Asai *et al.* (22) have found that *Pseudomonas fluorescens* forms both galactonic acid and 2-keto-galactonic acid, if the cells are incubated with galactose, but the subsequent steps in this metabolic chain seem to have been little studied. It is possible that Ophiostoma may oxidize galactose by a similar pathway, and that the organism under such conditions lacks sufficient glucose chains for normal growth.

Summary

The ascomycete *Ophiostoma multiannulatum,* which is a strictly aerobic organism, grows easily and with a short lag period on the following carbon sources: glucose, fructose, mannose, maltose, saccharose, ethanol and glycerol. Rhamnose, xylose, cellobiose, lactose, trehalose, raffinose, inositol, and acetate give less rapid growth with a more or less marked lag. Galactose, fucose, arabinose, sorbose, ribose, melibiose, melezitose, cellulose, starch, glycogen, pyruvate, citrate, succinate, fumarate, lactate, tartrate, and amino acids are not used as sole sources of carbon for growth in a synthetic medium.

The synthesis of β-glucosidase and β-galactosidase by the organism under various conditions of growth has been studied. Both enzymes are actively secreted into the medium during growth, but the synthesis of them is inhibited by glucose. The influence of different conditions of growth on the enzyme production has been studied, but owing to the difficulty of obtaining the enzymes quantitatively from the cells, no certain conclusions concerning the mechanism of synthesis can be drawn. The enzymes have been partly purified and shown to have pH-optima around 5.

The rate of respiration of Ophiostoma on a number of substrates has been

determined, and the results are briefly discussed in view of the current concept of the metabolism of carbon compounds. Worthy of note is the observation that galactose is readily oxidized, whereas this compound is not used as a sole source of carbon for growth.

References

1. Fries, N.: Über röntgen-induzierte physiologische Mutationen bei Ophiostoma multiannulatum (Hedgc. et Davids.) — Ark. f. Bot. 32 A, No. 8: 1. 1945.
2. Monod, J.: La technique de culture continue. Théorie et application. — Ann. Inst. Pasteur 79: 1. 1950.
3. v. Hofsten, B., v. Hofsten, A. & Fries, N.: Continuous liquid culture of the fungus Ophiostoma multiannulatum. — Exp. Cell Res. 5: 530. 1953.
4. Monod, J. & Cohn, M.: La biosynthèse induite des enzymes (adaptation enzymatique). — Adv. Enzym. 13: 67. 1952.
5. Davies, A.: Invertase formation in Saccharomyces fragilis. — J. Gen. Microbiol. 14: 109. 1956.
6. — Some factors affecting lactase formation and activity in Saccharomyces fragilis. — J. Gen. Microbiol. 14: 425. 1956.
7. Novick, A.: Growth of bacteria. — Ann. Rev. Microbiol. 9: 96. 1955.
8. v. Hofsten, A. & v. Hofsten, B.: Factors influencing cell division and morphogenesis of Ophiostoma multiannulatum. — Exp. Cell Res. (accepted for publication).
9. Ryan, F. J. & Schneider, L. K.: The consequences of mutation during the growth of biochemical mutants of E. coli. — J. Bact.: 58: 191. 1949.
10. Hestrin, S., Feingold, D. S. & Schramm, M. in Methods in Enzymology Vol. 1 (eds. Colovick, S. P. & Kaplan, N. O.) p. 241. Acad. Press Inc. New York 1955.
11. Umbreit, W. W., Burris, R. H. & Stauffer J. F.: Manometric Techniques and Tissue Metabolism. Burgess Pub. Co., Minneapolis 2nd ed. 1949.
12. Morton, A. G. & Broadbent, D.: The formation of extracellular nitrogen compounds by fungi. — J. Gen. Microbiol. 12: 248. 1955.
13. v. Hofsten, B.: Synthesis of β-galactosidase in Ophiostoma multiannulatum and some properties of the enzyme. — Nature 177: 844. 1956.
14. Cohn, M.: On the inhibition by glucose of the induced synthesis of β-galactosidase in Escherichia coli. — Enzymes: Units of Biological Structure and Function. (ed. Gaebler, O. H.) p. 41. Acad. Press, New York, 1956.
15. Boman, H. G. & Westlund, L. E.: Protein chromatography on an anion exchange resin. — Arch. Biochem. Biophys. 63: 217. 1956.
16. Tiselius, A., Hjertén, S. & Levin, Ö.: Protein chromatography on calcium phosphate columns. —- Arch. Biochem. Biophys. 63: 1956.
17. Alberty, R. A.: Enzyme kinetics. — Adv. Enzymol. 17: 1. 1956.
18. Herbert, D., Elsworth, R. & Telling, R. C.: The continuous culture of bacteria; a theoretical and experimental study. — J. Gen. Microbiol. 14: 601. 1956.

19. Gunsalus, I. C., Horecker, B. L. & Wood, W. A.: Pathways of carbohydrate metabolism in microorganisms. — Bact. Rev. 19: 79. 1955.
20. v. Hofsten, B.: Unpublished data.
21. Mandels, G. R.: Synthesis and secretion of invertase in relation to the growth of Myrotecium verrucaria. — J. Bact. 71: 684. 1956.
22. Asai, T., Aida, K. & Ueno, Y.: The oxidative bacteria. V. The production of 2-keto-galactonic acid from galactose. — J. Agr. Chem. Soc. Japan: 25: 625. 1951—52.

Initiation of Mitosis and Cell Division by Kinetin and Indoleacetic Acid in Excised Tobacco Pith Tissue

By

Nirmal K. Das, Klaus Patau, and Folke Skoog [1]

Departments of Botany and Pathology, University of Wisconsin
(Received September 26, 1956)

Introduction

The concept of the initiation of cell division in plants by endogenous growth factors goes back to Wiesner (1892), and especially to Haberlandt (1913) who showed that a substance derived from the phloem and diffusible through a thin film of agar induces cell division in the parenchymatous tissue of potato tuber. He also demonstrated that application of crushed cells promotes cell division (1914, 1921). Subsequent workers have confirmed Haberlandt's findings and have detected similar factors in various plant products especially in coconut milk (van Oberbeek et al., 1941; Caplin and Steward, 1948; Mauney et al., 1952; Shantz and Steward, 1955; see review by Gautheret, 1955), malt extract (Blakeslee and Satina, 1944), Datura embryo extract, almond meal and wheat germ (van Oberbeek et al., 1944), extract of milky stage corn kernels (Caplin and Steward, 1948) and carrot leaves (Wiggans, 1954). Skoog and Tsui (unpublished) and Jablonski and Skoog (1954) observed that tobacco pith cells with attached vascular strands when cultured on modified White's nutrient-agar medium with indoleacetic acid (IAA) added, undergo division and form callus. Similar effects can also be produced by placing segments of vascular tissue on or in contact with the pith tissue, or by planting a large number of stem segments on the medium around but not in contact with the pith pieces. Known growth promoting

substances such as adenine, a number of amino acids, various vitamins, and traumatic acid were tested but failed to induce cell division in excised pith tissue. This tissue, therefore, could be used for a bioassay by means of which a physiologically highly active compound called kinetin was finally obtained from deoxyribonucleic acid (DNA) as a starting material. Kinetin has been identified as 6-furfurylaminopurine and has been synthesized (Miller *et al.*, 1956).

The present paper will present results of the first part of a detailed cytological study of the effects of kinetin and IAA on the processes of mitosis and cytokinesis.

Material and Methods

Excised tobacco pith tissue, cultured *in vitro*, was used as experimental material. Cylinders of pith (8×3 mm) were taken from young internodes of ca. four months old and 2—3 ft. tall tobacco plants (*Nicotiana tabacum*, var. Wisconsin No. 38). The cylinders were cut lengthwise into two equal parts and cultured with the flat cut surface in contact with the nutrient medium. The basal medium was modified White's nutrient-agar medium (Miller and Skoog, 1953). Kinetin and IAA were incorporated in the medium in specified concentrations before autoclaving. The flasks containing these media were arranged in a randomized plot. Each flask contained 50 ml of medium and received two blocks of tissue picked at random. The cultures were kept in darkness at ca. 25° C. At certain intervals samples consisting of both blocks from one flask of each treatment were collected in a random manner and fixed in 1 : 3 acetic-alcohol for one day. This fixative was chosen because of its advantages for microphotometric measurements of nuclear DNA-contents (Feulgen) that were to be done on the same slides used for nuclear and cell counts. 50 μ thick sections were stained by the Feulgen method (Stowell, 1945) and sometimes counterstained with fast green. A few sections were stained with Harris' hematoxylin. Acetocarmine squashes were also made. In a few cases cells were isolated by digestion for two to three hours at 37° C in 4 % pectinase solution at pH 4 and stained with acetocarmine.

For counts of nuclei or cells, Feulgen stained sections were used exclusively. The sections were scanned in horizontal strips kept slightly separated so as to avoid counting a nucleus or a cell twice. A cut cell was scored as a cell if it contained at least one nucleus. In case of a cut nucleus the adjacent section was checked carefully in order not to count the same cell twice. A double count, however, might have resulted if two nuclei in one cell were separated by sectioning. In the relatively thick sections this could have occurred only rarely. Furthermore, the frequency of binucleate cells was always low and the nuclei in these were usually lying close together, except in enlarged old cells. In the few samples in which enlarged cells were found, the frequency of binucleate cells consequently may have been underestimated.

Nuclei were scored, with an oil immersion objective, as mitotic only if at least parts of chromosomes were clearly recognizable. This, as any criterion, leaves some uncertainty in the scoring of very early prophases and late telophases, and slight shifts of standard during a lengthy investigation may occur. To avoid systematic

errors from this source, the scoring was done in four 'runs', each of which covered the whole experiment. From each block of tissue to be studied three sections were scored at a time in the first run, three more sections in the second run, etc.

Results

A. *Effects on mitosis*

Kinetin alone clearly did not induce mitoses, as not a single one was found in a large number of sections taken at various times from tissues cultured on the basal medium with added kinetin concentrations of 0, 0.02, 0.05, 0.2, 0.8, and 1.6 mg/l. In contrast, IAA alone induced some mitoses (Figures 1, 2) in each of the two concentrations employed (0.2 and 2.0 mg/l), thus confirming earlier work by Naylor *et al.* (1954).

Addition of kinetin in combination with IAA caused a considerable increase in mitotic activity, the timing and magnitude of this effect being dependent on the concentration of kinetin (Figures 1, 2). Although the differences in mitotic counts between the two blocks from the same flask were often not statistically significant, in some cases they were highly significant (Table 1). This means that the biological variation may greatly exceed the random sampling variation. Therefore, statistical tests based only on the latter are not applicable, and no attempt will be made to evaluate differences between single samples. In the following analysis only major trends of the curves or repeatedly appearing effects will be considered.

After an initial period, which usually increased with the kinetin and IAA concentrations, mitoses occurred in rapidly increasing numbers (Figures
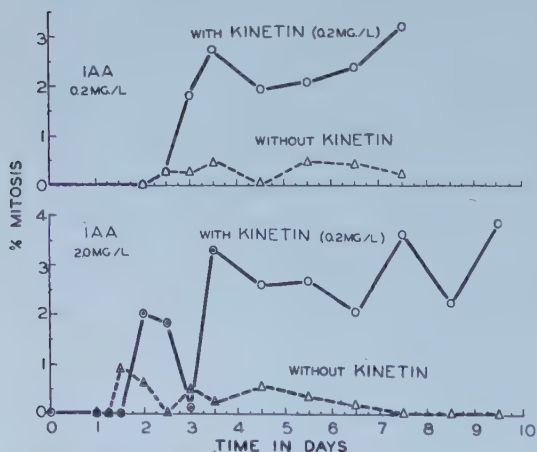

Figure 1. *Frequency of mitoses as a function of the duration of treatment with kinetin and IAA* (1st experiment). o, △: sample from one block of tissue; ⊙ △: mean per cent of samples from two blocks. Mean number of cells per sample: 616 (in all but five samples more than 400 cells each). Without IAA no mitoses were found at any time.
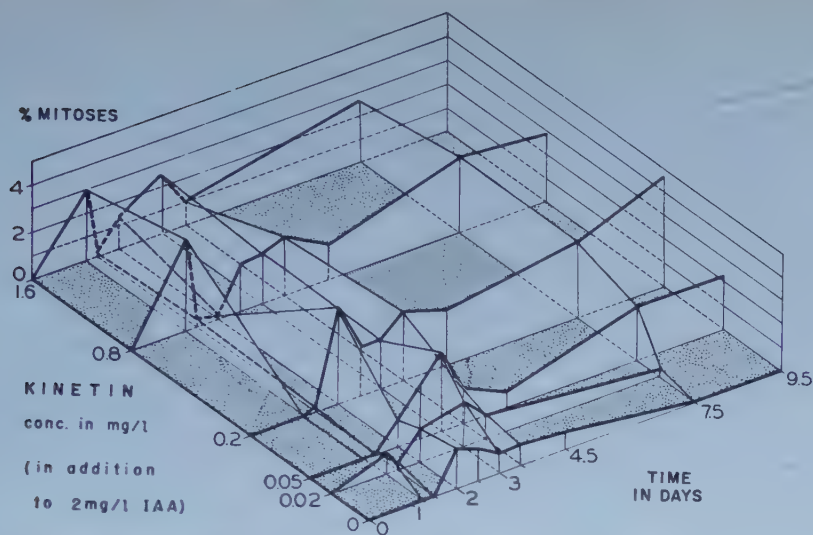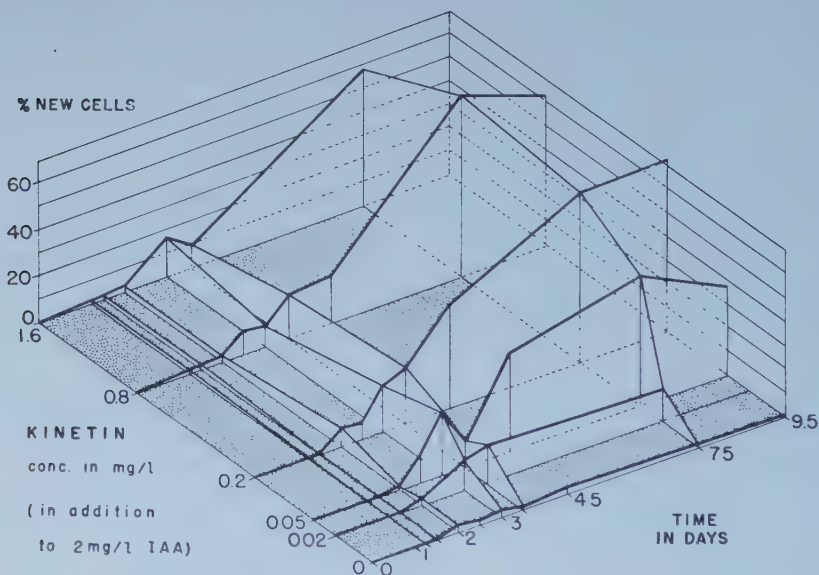
Figure 2. *Frequency of mitoses as a function of kinetin concentration and time of treatment* (2nd expt.). 49 samples (each from one block of tissue) of different combinations of concentration and time. Mean number of cells per sample: 590 (with two exceptions at least 450 cells each). IAA concentration 2.0 mg/l. This experiment was performed 5 months after that shown in Figure 1, which may account for any differences between corresponding curves in the two figures.

1, 2). Subsequently, the mitotic rate often, and perhaps always, decreased to a very low level. This drop was very pronounced with 2.0 mg/l of IAA and 0 and 0.2 mg/l of kinetin in the first experiment (Figure 1) and occurred in both tissue blocks (see Table 1). In the second experiment this drop occurred with 0.8 and 1.6 mg/l and apparently also with 0.2 mg/l of kinetin (Figure 2). In cases where no drop was apparent it might well have happened between

Table 1. *Variation between mitotic rates within pairs of tissue blocks from the same flask* (Experiment 1). Statistical significance (P) of difference tested by χ^2 after Yate's adjustment (test performed only, if smallest expected value larger than 3.7).

Duration of treatment in days	IAA (2 mg/1)			IAA (2 mg/1) + kinetin (0.2 mg/1)		
	% mitoses in the two blocks		P	% mitoses in the two blocks		P
0	0	0	—	0	0	—
1	0	0	— —	0	0	—
1 1/4	0	0	— —	0	0	—
1 1/2	0.18	1.59	0.0022	0	0	— —
2	0.49	— — 0.73	—	0.52	3.47	2×10^{-6}
2 1/2	0	0	—	2.11	1.54	0.560
3	0.22	0.78	—	0.15	0.10	—
3 1/2	0	0.46	—	2.27	4.30	0.052

times of sampling. After this drop the mitotic activity rose to a more or less constant level which was then maintained throughout the period under observation. This level increased with the kinetin concentration only up to about 0.2 mg/l (Figure 2) and was not greatly affected by raising the IAA concentration from 0.2 to 2.0 mg/l (Figure 1).

B. *Effects on cytokinesis*

The effects of various treatments on cytokinesis were studied by counting 'new cells' and binucleate cells. During the first three to four days new cells appeared almost exclusively in isolated pairs. At least in the inner regions of the blocks of tissue the new wall usually divided the parent cell into two very unequal parts. The inequality seemed to be due to the presence of a large vacuole that keeps the nucleus close to the cell wall even during mitosis (Figure 5 A) With treatments that induced continued cell division (Figures 3, 4) more and more of the new cells were present in clusters, each cluster being the result of repeated divisions in one old cell or a few neighboring

Figure 3. *Frequency of new cells as a function of kinetin concentration and time of treatment* (2nd expt.). 49 samples (each from one block of tissue) of different combinations of concentration and time. Mean number of cells per sampl : 533 (with two exceptions at least 440 cells each). IAA concentration 2 0 mg/l. The cells were scored under a high dry objective, those in Figure 2 under oil immersion. This accounts for the discrepency in the number of cells given in the two figures.

Figure 4. *Frequency of new cells as a function of the duration of treatment with kinetin and IAA* (1st expt.). o, △: sample from one block of tissue; ⊙: mean per cent of samples from two blocks. Mean number of cells per sample: 734 (in all but one sample more than 400 cells each)
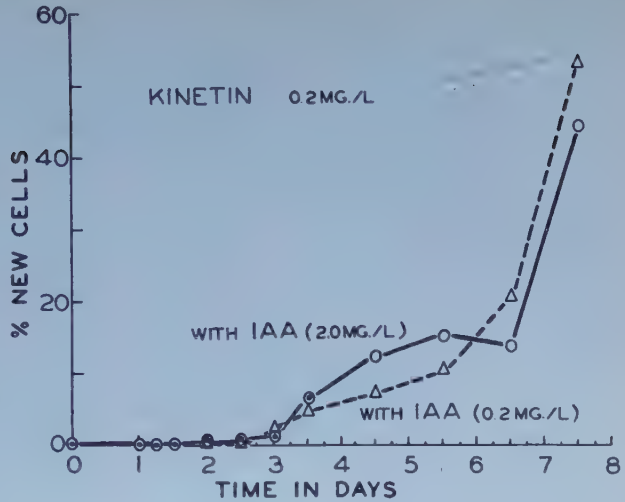
cells (Figure 5). In later periods many clusters had grown beyond the confines of the old cells, forming irregular patches or filaments of embryonic cells, (Figure 6 D).

New cells present in clusters cou d be counted fairly accurately, but the frequency of single pairs of new ells might have been greatly underestimated for the following reasons. Sister cells separated by sectioning were likely to be scored as two old cells In 50 μ thick sections this can have caused only relatively minor errors More serious errors no doubt arose from the fact that in the early stages most divisions occurred in the marginal parts of the blocks. Here the cells were very irregu arly shaped, which rendered the identification of many pairs of new cells extremely difficult. A comparison of ntegrated mitot c rates and counts of new cells indicated that, indeed, the new cells had been much less completely scored in the early than in the later periods. This counting error, of course, does not enter into comparisons of frequencies of new cells in differently treated samples from the same period. The frequencies of new cells were subject to similar biological variation as the mitotic frequencies. The statistically highly significant drop in the curve with a kinetin concentration of 0.05 mg/l (Figure 3) at three and one half days is a striking example. In biologically homogeneous material such a drop could not occur.

In tissue blocks treated with 2 mg/l of IAA some new cells were found. Their presence must be real because in tissues not supplemented with IAA an extensive search failed to reveal any such new cells. Samples from the 0.2 mg/l IAA concentration were not scored for new cells. The curve with kinetin concentration 0 in Fig. 3 indicates that the frequency of new cells
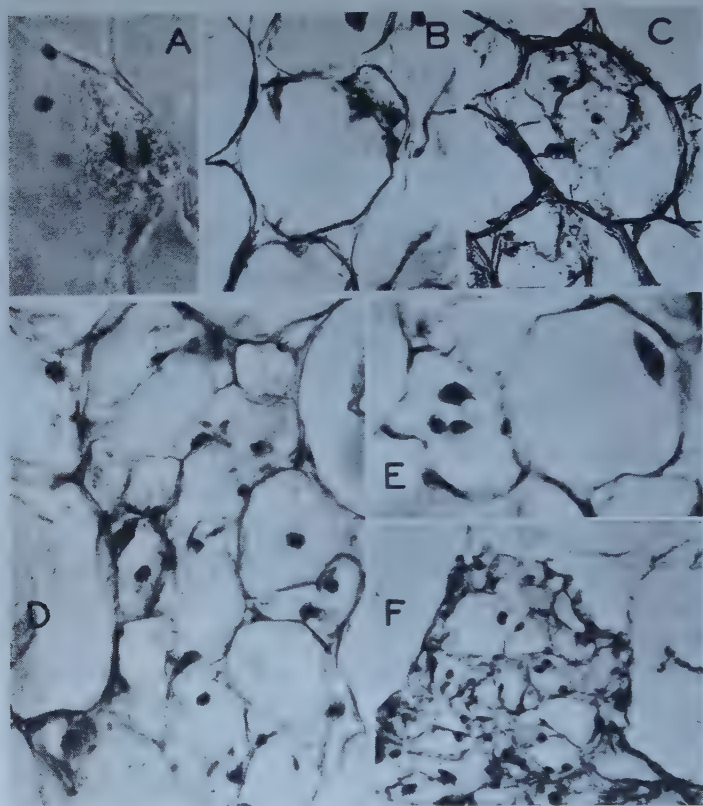
Figure 5. *Kinetin and IAA induced growth by cell division.* IAA: 2.0 mg/l; duration of treatment and kinetin concentration as stated in parentheses. *A.* Telophase with cell plate (3 days, 0.8 mg/l). *B.* Unequal division of an 'old' cell (4 1/2 days, 0.2 mg/l). *C.* Group of new cells within a parent cell (9 1/2 days, 0.8 mg/l). *D.* Cluster of meristematic cells (10 days, 0.2 mg/l). *E* Metaphase presumably leading to first division of an 'old' cell, adjacent divided cell (10 days, 0.2 mg/l). *F.* Patch of meristematic cells (10 days, 0.2 mg/l). A: Feulgen; B, C: Feulgen, fast green; D—F: Harris' hematoxylin. A: 345 ×; B—F 92 ×.

did not noticeably increase after the first two days, *i.e.* most of them were formed prior to this time, even though mitoses still occurred (Figures 1, 2). That most of these later mitoses were not followed by cytokinesis was further borne out by an increased incidence of binucleate cells (Table 3). Cells with more than two nuclei were not found. It has been stated already that in tissue with enlarged cells the frequency of binucleate cells tends to be underestimated. Cell enlargement starts after three to four days (see below). Therefore, the actual increase might have been larger than shown in Table 3.

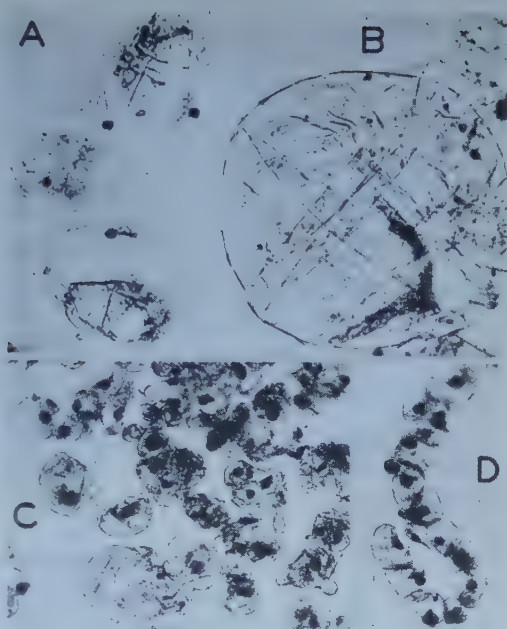Addition of kinetin to IAA containing medium not only increased the

Figure 6. *General biological effects of kinetin and IAA. A.* Normal pith cells (0 day, untreated). *B.* Highly enlarged cells found in between smaller cells (21 days, 2.0 mg/l of IAA). *C.* Embryonic (new) cells (21 days, 0.2 mg/l of kinetin+2.0 mg/l of IAA). *D.* Multicellular filament (21 days, 0.2 mg/l of kinetin+ 2.0 mg/l of IAA). A—C: Isolated by pectinase; all stained with acetocarmine. 45 ×.

mitotic activity but also must have caused practically every mitosis to be followed by cytokinesis (Figures 3, 4), because the frequency of binucleate cells was very low and showed no increase with time. Although this frequency was somewhat, but not significantly, higher than that in material not

Table 2. *Frequency of binucleate cells in relation to kinetin and IAA concentrations.* Data pooled from samples taken after 2, 2 1/2, 3, 3 1/2, 4 1/2, and 7 1/2 days of treatment.

Kinetin in mg/1	Expt. No.	Concentration of IAA in mg/1					
		0		0.2		2.0	
		Number of cells		Number of cells		Number of cells	
		total	binucl.	total	binucl.	total	binucl.
0 {	1	3,015	1	2,745	9	6,974	18
	2	—	—	—	—	3,350	7
0.02	2	—	—	—	—	2,542[1]	3
0.05	2	—	—	—	—	3,880	3
0.20 {	1	2,707	2	2,937	4	9,059	17
	2	—	—	—	—	3,859	5
0.80	2	—	—	—	—	3,716	4
1.60	2	—	—	—	—	2,448[1]	1

Group A: no IAA; group B: IAA present, no kinetin; group C: IAA and kinetin.
Test of homogeneity in group B: $\chi^2_2=0.82$; P=0.66.
Test of homogeneity in group C: $\chi^2_6=4.82$; P=0.56.

[1] No counts at 2 1/2 and 4 1/2 days.

Table 3. *Frequency of binucleate cells in relation to duration of treatment.* Groups A, B, and C as in Table 2. Data pooled from experiments with different concentrations which, according to Table 2, have not caused a noticeable intra-group heterogeneity.

Time in days	A: No IAA Number of cells			B: IAA alone Number of cells			C: IAA + kinetin Number of cells		
	total	binucl.	%	total	binucl.	%	total	bincul.	%
2, 2½	1,900	2	0.11	5,690	8	0.14	9,067	15	0.17
3, 3½	1,980	0	0	4,287	11	0.26	10,732	15	0.14
4½, 7½	1,842	1	0.05	3,092	15	0.49	8,642	7	0.08
	5,722	3	0.05				28,441	37	0.13

Significance of increase with time in group B: $\chi^2_2 = 9.17$; $P = 0.01$.

Significance of difference B—C at 4½, 7½ days: $\chi^2_1 = 17.78$ (after Yate's adjustment); $P = 2.5 \times 10^{-5}$.

Test of significance of difference A—C (overall): $\chi^2_1 = 1.84$ (after Yate's adjustment); $P = 0.17$.

supplied with IAA, it was considerably lower than the frequency of binucleate cells reached after four and one half days of treatments with IAA alone (Table 3). When the frequencies of binucleate cells from treatments with and without kinetin are related to those of new cells (compare Table 3 with Figures 3, 4) the enhancing effect of kinetin on cytokinesis is obvious. The frequency of new cells, as the mitotic rate, was not greatly affected by increasing the concentration of kinetin above 0.2 mg/l.

C. *General biological effects*

Our observations on the general biological effects of IAA on pith tissue agree with earlier reports (Jablonski and Skoog, 1954; Naylor, Sander, and Skoog, 1954). Cell enlargement began after about three to four days of treatment with IAA alone (2 mg/l). Some abnormally lobed nuclei appeared, a few of which seemed to be fragmented later on. With prolonged treatment many cells became greatly enlarged (Figure 6 B) and some of these produced 'pseudopod' like out-growths. Subsequently tissue degeneration set in, but apparently later than in cultures not supplied with IAA. In tissues treated with kinetin these same responses to IAA were found in cells which did not divide.

Discussion

In pith tissue cultured without IAA no mitoses were found, regardless of the presence or absence of kinetin in the medium. IAA without kinetin induced some mitoses. Some cell division also occurred but mainly only after

the first mitoses; most of the later mitoses merely produced binucleate cells. Kinetin in combination with IAA greatly increased the frequency of mitoses and, furthermore, caused virtually all of them to be followed by cytokinesis. It is evident from the above that in presence of IAA kinetin not only induces mitosis to set the stage for cell division, but that it also stimulates the latter process itself. This does not mean, of course, that either process is brought about by a direct action of kinetin.

The term kinin has been suggested as a generic name for all substances which promote cell division in a manner similar to that of kinetin (Okumura *et al.*, 1955; Strong, 1955; Miller *et al.*, 1956). Our observations suggest that kinin is a necessary factor for mitosis as well as for cytokinesis in the pith tissue. In our experiments this tissue did not contain effective levels of auxin. It would seem that a higher concentration of kinin is required for cytokinesis than for mitosis, because in tissue treated with IAA alone, mitoses (although in decreasing frequency) were still found after the more or less complete cessation of cell division. The postulated decrease in natural kinin in pith tissue cultivated in presence of IAA could be due either to its being metabolized in the cell, or to its loss into the medium, or both. That the active compound in vascular tissue may diffuse out into the medium has been shown by Jablonski and Skoog (1954).

Differences in endogenous kinin content relative to need would readily account for an apparent discrepency between our results and those of Naylor *et al.* (1954) who found IAA to induce mitoses but not cell divisions. This would be expected if in their pith material the natural kinin concentration was relatively lower than in the pith tissue we used, *i.e.* below the threshold requirement for cytokinesis but still above that for mitosis.

With increasing concentrations of IAA and kinetin in the culture medium the time of treatment after which the first mitosis appeared was somewhat shortened. We presume that this reflected a shortening of the period required to reach an effective concentration in the cell. The striking dip in the rate curve during the first period of mitotic activity seems to indicate that all pith cells which were 'ready' were triggered into mitoses within a rather short period of time. During the later stages the average mitotic frequency was not noticeably affected by increasing the IAA or kinetin concentrations beyond 0.2 mg/l. Even in very late samples (four weeks), a great number of 'old' cells were still present. The fact that these cells had failed to divide may indicate that other substances which had become unevely distributed in the tissue were now limiting factors. It is of course not excluded that some cells had lost irreversibly their mitotic potentialities.

One essential prerequisite for a complete mitosis is the duplication of the nuclear DNA-content which presumably indicates reproduction of chromo-

some strands. On the other hand, DNA duplication is not invariably followed by mitosis; in a sense the two processes are independent of each other (Patau and Swift, 1953). Extensive microspectrophotometric DNA determinations (Feulgen) in our material are being prepared for publication. It was found that after about one day of treatment with 2 mg/l of IAA, with or without added kinetin, a considerable fraction of nuclei underwent DNA synthesis prior to the onset of the mitotic wave. It was indicated that kinetin without IAA also stimulated DNA synthesis and that both auxin and kinin are required for mitosis itself. It should be mentioned that increases in DNA-contents determined by the Ogur and Rosen method, as those reported by Silberger and Skoog (1953), must be considered as uncertain because of limitations in the use of the method with plant tissues as shown by Haber (1956).

It appears from the present results and additional data to be published that the full cycle of cell multiplication — DNA synthesis, mitosis, and cyto-kinesis — requires both auxin and kinin. When one of these two factors is present in abundance the other may become limiting for more than one of these processes. Our work thus lends support to the concept that in plants, growth by cell division is regulated by a proper balance between endogenous growth factors.

Summary

Frequencies of mitoses, new cells, and binucleate cells have been determined in excised pith tissue cultivated on nutrient medium with various concentrations of kinetin or indoleacetic acid (IAA) or both together.

Kinetin, in combination with IAA, induced many mitoses which were virtually always followed by cytokinesis. These activities continued throughout the period of observation (about one month) leading to patches of small meristematic cells between undivided cells.

Without IAA no mitoses took place. With IAA alone some mitoses occurred probably due to the presence of small amounts of natural kinin. Only few of these mitoses were followed by cell division.

References

Blakeslee, A. F. & Satina, S.: New hybrids from incompatible crosses of Datura through culture of excised embryos on malt media. — Science 99: 331. 1944.

Caplin, S. M. & Steward, F. C.: Effect of coconut milk on the growth of explants from carrot root. — Science 108: 655. 1948.

Gautheret, R. J.: The nutrition of plant tissue cultures. — Annual Review of Plant Physiology 6: 433. 1955.

Haberlandt, G.: Zur Physiologie der Zellteilung. — Sitz.ber. k. preuss. Akad. Wiss.: 318. 1913.

— Zur Physiologie der Zellteilung. — Sitz.ber. k. preuss. Akad. Wiss.: 1096. 1914.

— Wundhormone als Erreger von Zellteilungen. — Beitr. allg. Bot. 2: 1. 1921.

Haber, A. H.: 1. On the estimation of nucleic acids in tobacco pith. — Ph.D. Thesis, University of Wisconsin. 1956.

Jablonski, J. R. & Skoog, F.: Cell enlargement and cell division in excised tobacco pith tissue. — Physiol. Plant. 7: 16. 1954.

Mauney, J. R., Hillman. W. S. Miller, C. O., Skoog, F., Clayton, R. A., & Strong, F. M.: Bioassay, purification, and properties of a growth factor from coconut. — Physiol. Plant. 5: 485. 1952.

Miller, C. O. & Skoog. F.: Chemical control of bud formation in tobacco stem segments. — Amer. J. Bot. 40: 768. 1953.

— — Okumura, F. S., von Saltza, M. H. & Strong, F. M.: Isolation, structure and synthesis of kinetin, a substance promoting cell division. — J. Amer. Chem. Soc. 78: 1375. 1956.

Naylor, J., Sander, G. & Skoog, F.· Mitosis and cell enlargement without cell division in excised tobacco pith tissue. — Physiol. Plant. 7: 25. 1954.

van Oberbeek. J., Conklin, M E., & Blakeslee, A. F.: Factors in coconut milk essential for growth and development of very young Datura embryos. — Science 94: 350. 1941.

— Sui, R., & Haagen-Smit, A. J : Factors affecting the growth of Datura embryos in vitro. — Amer. J. Bot. 31: 219. 1944.

Okumura, F. S., von Saltza, M. H., Strong, F. M., Miller, C. O., & Skoog, F.: Kinetin analogues. — Abstract, 128 Mtg. Amer. Chem. Soc. Minneapolis, Minn. Sept. 1955.

Patau, K. & Swift, H.: The DNA-content (Feulgen) of nuclei during mitosis in a root tip of onion. — Chromosoma, Bd. 6, S.: 149. 1953.

Shantz, E. M. & Steward, F. C.: The identification of compound A from coconut milk as 1,3-diphenylurea. — J. Amer. Chem. Soc. 77: 6351. 1955.

Silberger. J., Jr., & Skoog, F.: Changes induced by indoleacetic acid in nucleic acid contents and growth of tobacco pith tissue. — Science 118: 443. 1953.

Stowell, R. E.: Feulgen reaction for thymonucleic acid. — Stain Tech. 20: 45. 1945.

Strong, F. M.: Kinetin and kinins. — Lect. Southwest Regional Mtg., Amer. Chem. Soc., Houston, Texas. Dec. 1, 1955.

Wiesner, J.: Die Elementarstruktur und das Wachstum der lebenden Substanz. — Wien, 1892.

Wiggans, S. C.: Growth and organ formation in callus tissues derived from Daucus carota. — Amer. J. Bot. 41: 321. 1954.

Chemistry of Arylboric Acids. VI. Effects of Arylboric Acids on Wheat Roots and the Role of Boron in Plants

By

Kurt Torssell

Institute of Organic Chemistry and Biochemistry, University of Stockholm,
Stockholm, Sweden and Botanical Laboratory, University of Lund,
Lund, Sweden
(Received September 27, 1956)

Introduction

The discovery of indole-acetic acid as a natural growth-regulating factor gave the signal for a search for other active compounds of similar constitution. It was soon established that derivatives of indolenaphthoxy- and phenoxyacetic acids were especially active. Some simple derivatives of benzoic acid, *e.g.* 2,6-dichlorobenzoic acid, also showed growth-regulating properties similar to those of the naturally occuring auxin.

In a series of papers the present author (11) has described the syntheses and properties of a number of new organic boron compounds, which have the general formula $RB(OH)_2$, where R represents a phenyl group carrying substituents in different positions.
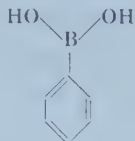
Phenylboric acid.

These compounds have a structure similar to that of the benzoic acid derivatives — the dihydroxyboron-group has, like the carboxyl-group, acidic properties — and there are consequently reasons to suppose that compounds

of this type may have growth-regulating properties. It was therefore of interest to test some selected compounds for their auxin activity. The Avena straight growth test and the Triticum root test were performed. Root growth as well as real cell elongation was measured.

Experimental

Wheat root test. The micro method employed was worked out by Burström *et al.* Eroica wheat (Weibull) was soaked for 24 hours in distilled water freed from heavy metal ions and germinated for two days in the dark at 22° in Petri dishes on wet filter paper. Seedlings with their roots about 1.5 cm in length were planted on perforated plastic plates in 50 ml beakers containing 5 ml nutrient solution and the substance to be tested. Each beaker contained 2 seedlings and for every concentration 10 plants were used. After 24 hours at 22° in the dark the root growth was measured and in some experiments the length of the epidermal cells also (Burström, 3). The root and cell elongation of the treated seedlings are expressed as per cent of the control.

The *Avena test* was performed according to Fransson and Ingestad (6).

Determination of amylose retrogradation in the presence of boric acid and phenylboric acid. Maize amylose (500 mg) was suspended in methanol (10 ml) and poured into boiling water (90 ml). The solution was boiled until the methanol had been volatilized, filtered through a glass filter and diluted to 100 ml. The following three solutions were prepared. *A.* Amylose solution (20 ml) + water (5 ml). *B.* Amylose solution (20 ml) + boric acid (5 ml, 5×10^{-2} M). *C.* Amylose solution (20 ml) + phenylboric acid (5 ml 5×10^{-2} M). The pH of the solutions is 5—5.5. The final concentration of boric acid and phenylboric acid is 10^{-2} M. Three analogous solutions buffered to pH 8.2 were also prepared. The intensity of the scattered light was measured at intervals with a *Leifo-Photometer*. Figure 3 shows the time course of the retrogradation of maize amylose. The intensity of the scattered light increases as the number of colloidal aggregates increases.

Results

When tested, the plants showed quite different behaviour from that expected. The arylboric acids possess a very strong root growth promoting effect in concentrations between 10^{-6} M and 10^{-4} M. At still higher concentrations they are toxic and at concentrations $< 10^{-7}$ M no effect was observed. Some compounds are toxic even at concentrations of about 10^{-5} M e.g. naphthylboric acid (Figure 1. number 3.) and o-hydroxymethyl-phenylboric acid (number 21.). The following twenty-four arylboric acids have been tested:

1. Phenylboric acid
2. *p*-Tolylboric acid

3. Naphthylboric acid
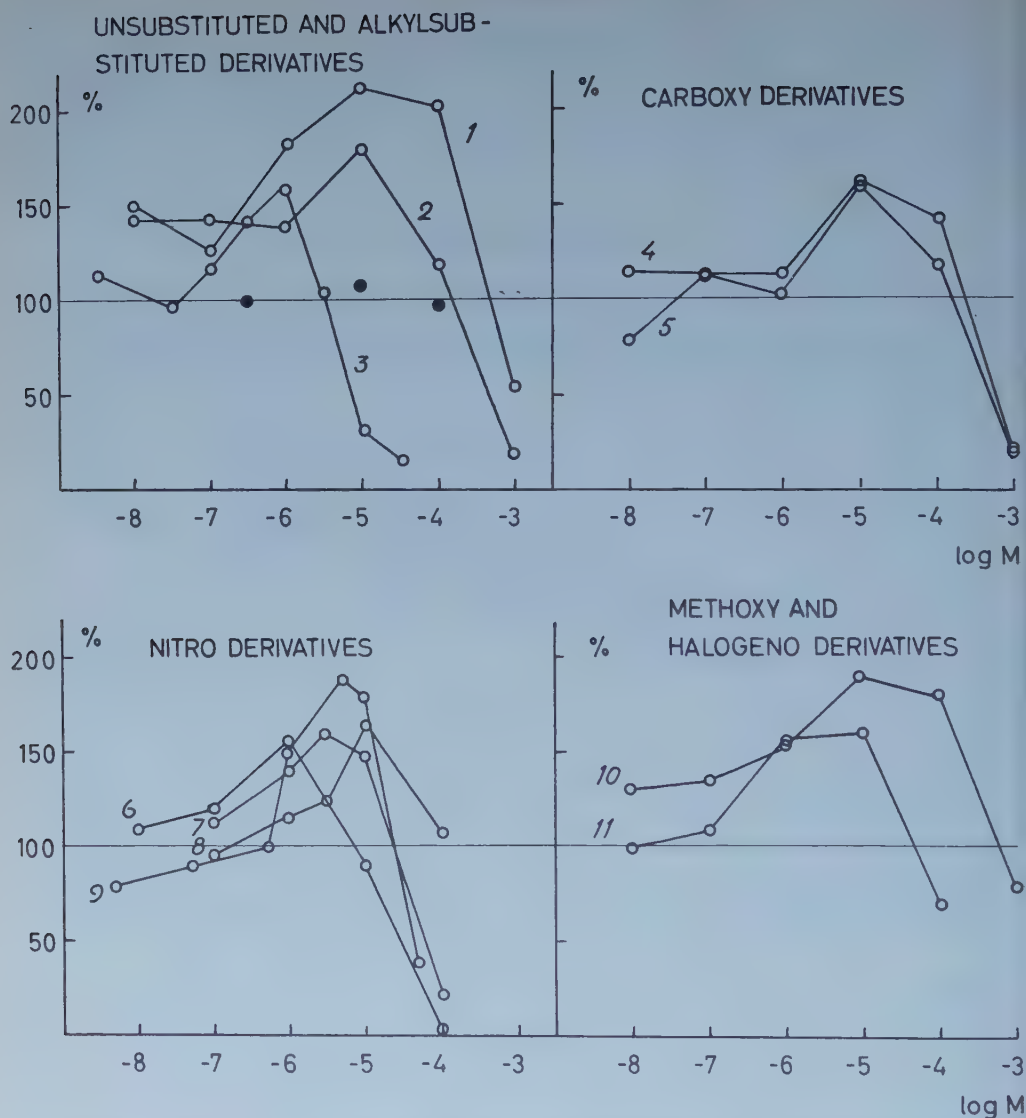4. 3-Carboxy-phenylboric acid

Figure 1 (numbers 1—11). *The action of various arylboric acids on root growth of wheat. The root growth is expressed as per cent of the control (100 %).*

5. 4-Carboxy-phenylboric acid
6. 2-Methyl-3,5-dinitro-phenylboric acid
7. 3-Nitro-phenylboric acid
8. 2-Nitro-phenylboric acid
9. 4-Nitro-phenylboric acid

10. 4-Methoxy-phenylboric acid
11. 4-Chloro-phenylboric acid
12. 3-Carboxy-5-nitro-phenylboric acid
13. 2-Nitro-4-carbomethoxy-phenyl-boric acid
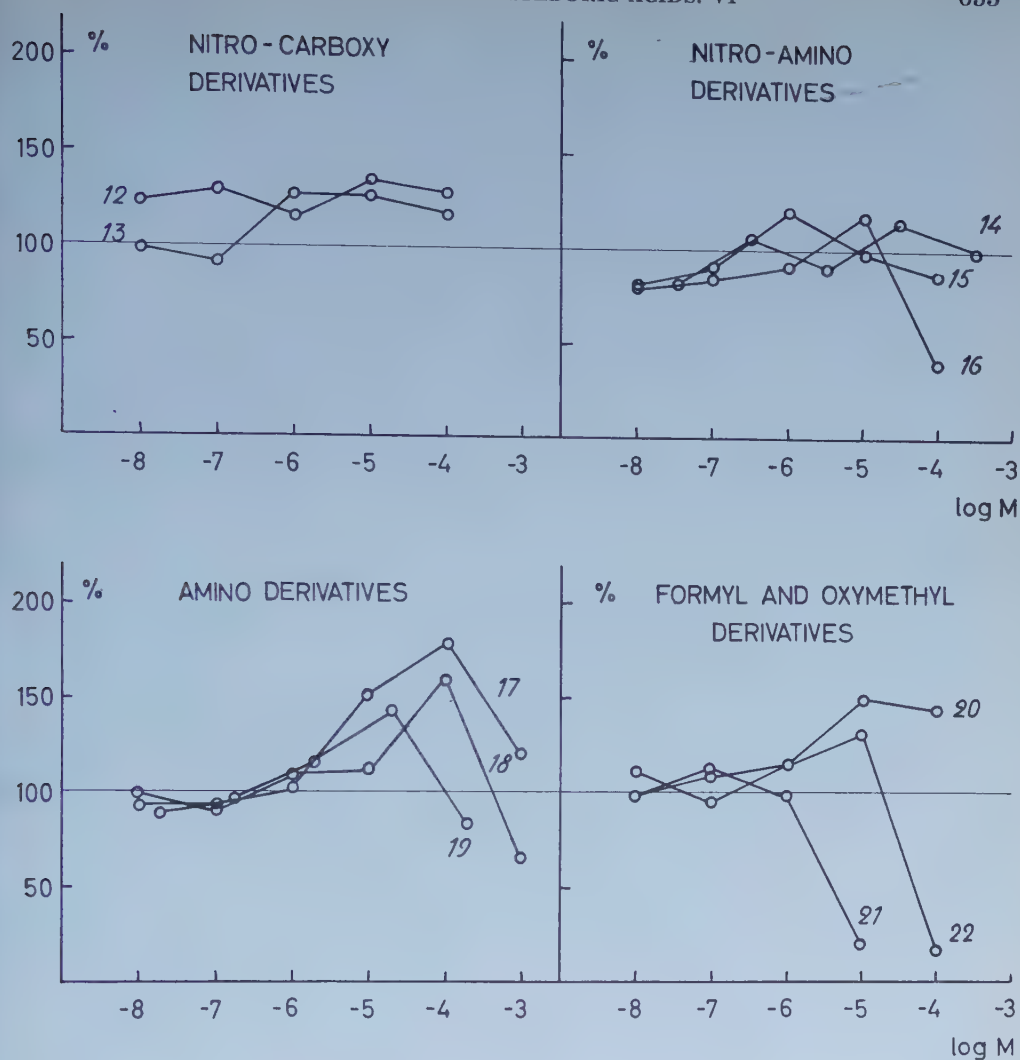
Figure 1 (numbers 12—22).

14. 2-Nitro-4-amino-phenylboric acid
15. 2-Nitro-5-amino-phenylboric acid
16. N,N'-*bis* [2-nitro-phenylboric acid(4)]-urea
17. 3-Acetylamino-phenylboric acid
18. 4-Acetylamino-phenylboric acid
19. 4-Dimethylamino-phenylboric acid

20. 3-Formyl-phenylboric acid
21. 2-Hydroxymethyl-phenylboric acid
22. 2-Formyl-phenylboric acid
23. 4-Carbethoxyamino-phenylboric acid
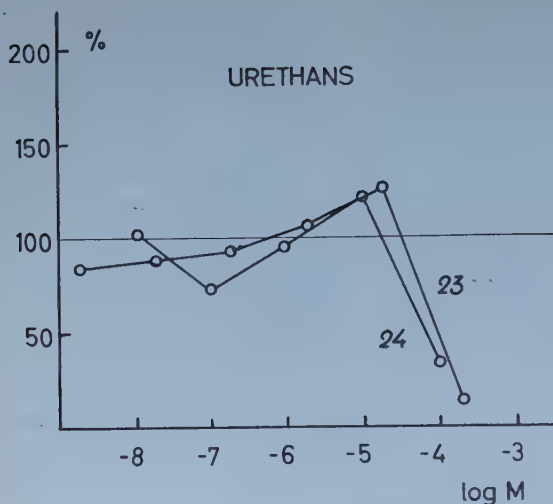24. 3-Carbethoxyamino-phenylboric acid

Figure 1 (numbers 23—24).

This material permits, of course, only rough conclusions about the rela
tionship between structure and activity. Figure 1 shows the root grow h,
expressed as per cent of the control (100 per cent), as a function of the con
centration of the tested substance. All curves have their maxima at a c n
centration of about 10^{-5} M. The most active arylboric acids are those con-
taining lipophilic groups attached to the nucleus (hydrogen, alkyl halo en
methoxy, nitro). The simple phenylboric acid shows the highest activity.
210—220 per cent while 4-methoxy-phenylboric acid and 4-nitro phenylbor
acid cause a root elongation of 190—200 per cent (numbers 10 and 9

Arylboric acids containing hydrophilic groups *e.g.* nitroam'nes,   itro-
carboxylic acids, carbamic acids, hydroxymethyl- and formyl derivatives are
inactive or very slightly act ve The acetylated amines 4 dimethylamino-
phenylboric acid and 3- and 4-carboxy-phenylboric acids are active As a
general rule it may be said that hydrophilic groups diminish or abolish the
activity.

In the standard test employed he elongation of the roots is measured after
one day but after 2 days the in rease in growth rate is unchanged or only
slightly increased. Table 1. In one experiment the wheat seedlings were
allowed to grow for 10 days in an aerated, continuously flowing nutrient
solution containing 10^{-5} M phenylboric acid in order to investigate whether
the effect continues for a lon er time. During this time the roots continued
growing at about the same rate in agreement with the one day test and were
almost twice as long as those of the control. The development of the aerial
parts of the plant was a little inhibited.

In order to ensure that the cell elongation effects are not an effec of boric

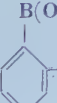Table 1. *Effect of arylboric acids on the growth of wheat roots after various times.*

Compound	Concentration M	Root growth mm.		Root growth as % of the control	
		1 day	2 day	1 day	2 day
Control	—	9.4	10.6	100	100
Phenylboric acid	0.5 · 10⁻⁵	15.9	18.4	169	173
4-Methoxy-phenylboric acid	10⁻⁵	18.4	23.2	196	218
» »	10⁻⁴	15.0	16.2	160	156

acid possibly split off from an organic boron compound a control was run with increas ng concentrations of boric acid in the nutrient solution. No effect could detected (see Figure 1, filled rings).

An increase of roo growth depends either on increased cell multiplication or on an increased cell elongation, or of course on a combination of the two effects. It is possible to separate these effects by measuring both the root growth and length of the epidermal cells of treated and control roots. Table 2 shows the cell elongation and root elongation expressed as per cent of the control. The figu es in the two columns agree fairly well, which means that the root elongation depends solely on a cell stretching effect produced by organic boron compounds.

Compounds class fied as anti-auxins *e.g* phenoxy-*iso*butyric acids stimulate the cell elongation of roots but have no pronounced effect on shoots. It was

Table 2. *Comparison between cell elongation and total root growth of wheat roots treated with arylboric acids.*

Compound	Concentration M	Cell elongation % of the control	Root growth % of the control	Cell length μ
Control	—	100	100	250
B(OH)₂ (phenyl)	10⁻⁶	166	160	414
B(OH)₂ (NHAc-substituted)	10⁻⁴	161	159	402
B(OH)₂ —NO₂	10⁻⁵	178	164	444

Figure 2. *The action of some arylboric acids on the elongation of Avena coleoptiles.*

therefore of interest to compare the effects of arylboric acids on the different parts of the plant. The usual *Avena* test was performed. Figure 2 shows the stretching of isolated coleoptile sections, expressed as per cent of the control (100 per cent), as a function of the concentration of the tested substance. High concentrations $> 10^{-5}$ M decrease the cell elongation. Low concentrations $< 10^{-5}$ M have only a slightly positive effect (4-methoxy-phenylboric acid) or no effect (the other four compounds tested).

Since the completion of this work, certain papers of Caujolle and Bergal (4, 5) which deal with the plant physiological effects of phenylboric acid, have come to the attention of the author. They have observed the root growth promoting properties of phenylboric acid and in agreement with our results found that naphthylboric acid was more toxic to the seedlings and less active than phenylboric acid.

Discussion. The mechanism of cell elongation and the role of boron

There are only a few compounds known that possess a strong promoting effect on root growth. Burström (2) found that some derivatives of α-phenoxy-isobutyric acid have a strong positive effect on root growth the most active

compounds giving rise to a root elongation of about 200 per cent. It was concluded that this effect depends upon an antagonistic action on the natural auxin. However, the morphologic effects of the *iso*butyric acid derivatives are different from those caused by the arylboric acids. The development of root hair is unaffected or slightly promoted by the latter compounds, even at maximal elongation, while it is inhibited by the *iso*butyric acids.

The epidermal cells of wheat roots treated with arylboric acids are narrower than the normal ones, the cell walls are very thin and the roots are limp and weak, which often makes it difficult to measure their length.

The position and the type of substituent in the nucleus of the synthetic auxins have a large effect on the activity (10, 12, reviews), but it is obvious that this is much smaller with the organic boron compounds. No derivatives of benzoic acid containing a lipophilic substituent *e.g.* a hydroxyl or amino group has been found to be active. Further in contrast to the general activity of the different arylboric acids there are rigorous sterical requirements for auxin activity in arylalkyl-, aryloxyalkyl and indole-alkylcarboxylic acids. It therefore seems unlikely that they act in the same way as the auxins or anti-auxins, even if the dihydroxyboron group plays the same role as the carboxyl group. Benzoic acid has no growth-regulating effects while phenylboric acid has a very strong activity. A considerably more attractive explanation of the effects produced by the organic boron compounds lies in the assumption that the dihydroxyboron group possess quite outstanding properties, which in some way regulate the growth of the cells — properties not coupled with auxin effects.

In discussing the effects of organic boron compounds on plant tissues, the foremost question is the mechanism of cell elongation and the role of these compounds in that mechanism. Gauch and Dugger (7) have pointed out the role of boron in the translocation of sugars. By labelling sugars with radioactive carbon they have found that the uptake and transport of sugars in normal plants is more rapid than in boron deficient plants. It is a well-known fact that boric acid forms complexes with polyalcohols. The equilibrium between the components and the complex in neutral and acid solution is strongly displaced in the direction of the components (to the left) and in alkaline solution towards the complex (Böeseken, *et al.* 1).

$$D + B^- \rightleftarrows BD^-$$
$$BD^- + D \rightleftarrows BD_2^-$$
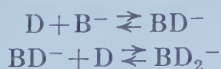
B^- represents the borate ion and D a polyalcohol.

Gauch and Dugger (7) suppose that this ionised complex passes more readily through the plasma than the sugar molecules themselves. It is also possible that the ions are subject to an active salt transport. Another explana-

tion was also postulated, *viz.* that boric acid is a constituent of the plasma membrane and the sugars are transported through the "barrier" by a temporary coupling with the borate ion. The sugar is then dissociated off on the other side of the membrane. Phenylboric acid forms complexes with sugars to a higher degree than boric acid but phenylboric acid can only bind one sugar molecule (Torssell, 11).
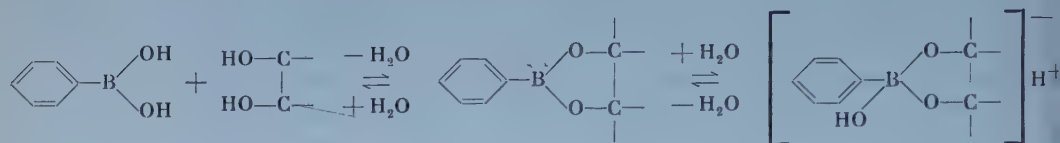
The pH of a water solution of phenylboric acid decreases strongly when mixed with *e.g.* laevulose. From the change of pH the complex constant can be calculated (11). A large change of pH means strong complex formation. When the inactive 2-nitro-4-carboxy-phenylboric acid was tested, the change of pH was very small. This indicates that the activity of a compound parallels its ability to form complexes with sugars.

The lipophilic phenyl group may facilitate the transport of the complex through the membrane, which is assumed to be composed mainly of a lipidic layer with areas of hydrophilic constituents (proteins) in a mosaic structure. Phenylboric acid is soluble in benzene, boric acid is insoluble. When applying the alternative view of boron action to the arylboric acids, it is quite clear that phenylboric acid derivatives with an lipophilic group could be incorporated more easily than boric acid in the plasma membrane and thus take part in the sugar translocation to a higher degree. The statement above that phenylboric acids containing lipophilic substituents are generally more active than those containing hydrophilic substituents supports this view. The capacity of the different arylboric acids to form complexes with sugars is of decisive significance and it is probable that a combination of "capacity" and "lipophility" determines the activity of the compounds. According to this theory a derivative that does not form complexes with sugars will not possess any activity and in agreement with this it has been found that the inactive 2-nitro-4-carbomethoxy-phenylboric acid does not form any appreciable amount of complexes. Thus it is concluded that phenylboric acid or its derivatives may promote the transport of sugars through membranes but it is rather unlikely that this circumstance only can have such an overwhelming effect on root growth. The addition of sugar to the nutrient solution has only a slightly positive effect on cell elongation. The effect of boric acid and phenylboric acid on the transport of sugars through the plasma membrane will be studied further and reported in a forthcoming paper.

Some workers have reported an effect of boric acid on some enzyme systems in plants, while other enzymes have been found to be unaffected. For a review of the physiological action of boron see Gauch and Dugger (8). The cell elongating effects of arylboric acids might be explained by an effect on an enzyme (some type of phosphorylase) regulating the synthesis of cell wall material (polysaccharides). However, in an investigation by the present author (11) it was found that phenylboric acid in concentrations $\leq 10^{-3}$ M is without effect on starch phosphorylase, α-amylase invertase and the fermentative activity of baker's and brewer's yeast. From the chemical point of view boric acid and phenylboric acid are unreactive. They can only combine with polyols to form very loose complexes (see above) The conditions *in vitro* are of course not completely comparable to those *in vivo* but nevertheless it seems reasonable to assume that the activity of arylboric acids is not due to an effect on an enzyme

The new theory presented below explains fairly well the effects of aryl-boric acids and the mechanism of cell elongation; the role of boron (boric acid) in plants comes thereby in a new light.

The cell wall is mainly built up of cellulose and pectins. When aged the rigidity of the wall increases with diminishing tensibility as a consequence. This change depends upon an increasing orientation of the polysaccharide chains, which undergo a type of "crystallisation" At points where complexes with arylboric acids are formed, this bonding of the chains by van der Waal's forces or by hydrogen bonds is precluded partly because of steric hindrance and partly because of repulsion caused by the ionised complexes, but a slow crystallisation will take place at these points as the complexes gradually dissociate. In this way the stiffening of the cell wall will be delayed and a high tensibility maintained, which will result in abnormal cell growth.

The action of boron (boric acid) in plants can be explained in the same way. It is stated that its site of action is at places characterised by a rapid rate of growth *e.g.* root tips, where a rapid synthesis of cell wall material takes place The complexes between boric acid and carbohydrates control the deposition of oriented cellulose micells and the accompanying stiffening of the cell wall. As a consequence of this view it may be assumed that in boron-deficient plants a rapid stiffening of the cell wall takes place, which prevents the normal stretching of the cell. On the other hand, when the normal stabilisation of the cell wall is prevented by arylboric acids, it will result in an increased cell elongation. The rigidity of the cell wall, regulated by boric acid, may thus be a limiting factor for the root cell growth.

The high activity of the arylboric acids might depend upon·

1. the lipophilic group permitting the molecule to permeate lipid layers.
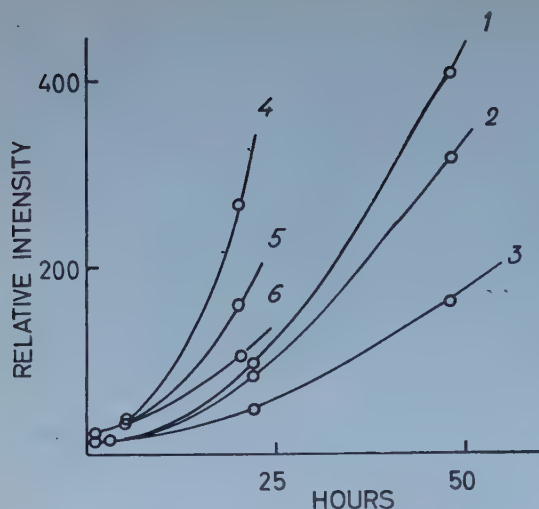
Figure 3. *Retrogradation of maize amylose in presence of boric acid and phenylboric acid at two different pH values.* Curves 1, 2, 3 at pH 5—5.5; curves 4, 5, 6 at pH 8.2. Curves 1 and 4 represent the control; curves 2 and 5 runs with 10^{-2} M boric acid added and curves 3 and 6 with 10^{-2} M phenylboric acid added.

2. the ability to form complexes. It is obvious that the "crystallisation" will be more delayed if more ionised complexes are formed.

In connection with these suggestions it may again be pointed out that the aryl group prevents the formation of a complex containing two ligands, while boric acid is able to link two chains together and this means that an arylboric acid is more effective than boric acid in preventing the "crystallisation".

This hypothesis is supported by the following experiment.

The retrogradation of amylose may serve as an analogy to what happens in the cell wall during growth. If amylose *i.e.* the unbranched part of starch is brought into water solution, it will after some hours have undergone retrogradation. This phenomen can be explained as a type of "crystallisation". The forces acting between different chains orientate them and when an aggregate grows large enough it will begin to separate from the solution. It is now found that boric acid and phenylboric acid delay the retrogradation, which can be explained according to the ideas developed above. Figure 3 shows the retrogradation, [expressed as an arbitrary unit], as a function of time (hours) at two different pH values. Curves 1 and 4 represent the control, curves 2 and 5 runs with 10^{-2} M boric acid added and curves 3 and 6 with 10^{-2} M phenylboric acid added. It is rather suggestive that phenylboric acid is more active than boric acid.

The method used to follow these changes is based on nephelometry and is only applicable until the aggregates grow so large that they precipitate. The experiment was stopped when the amylose in the control test precipitated.

By this time the retrogradation in 10^{-2} M phenylboric acid had only progressed half way to completion.

Many symptoms of boron deficiency. can readily be interpreted by this theory, *e.g.* the abscission of flowers, buds and fruits — all organs in rapid growth. There are numerous reports (8, review) that boron deficiency results in an accumulation of sugars in the leaves of plants. The changes in the properties of the cell wall, induced by boron deficiency, may react on the transport of metabolic products in plants. Johnston and Dore (9) suggested that this accumulation depends on a disintegration of the phloem, which fits in very well with the theory outlined.

Summary

The effects of arylboric acids on the growth of roots were investigated. It was found that these compounds promote root growth very strongly. It is an effect of cell elongation since the cell multiplication is left unaffected. Cell elongations of more than 200 per cent are found. The most active compound is phenylboric acid and 4-methoxy-phenylboric acid. Twentyfour compounds have been tested and relationships between structure and activity are shown. They are without effect in the *Avena* coleoptile test.

On the basis of these findings the mechanism of boric acid and phenylboric acid action in plants is discussed. The chemical behaviour of the compounds is compared with their suggested action in the cell elongation mechanism. Many symptoms of boron deficiency can be explained by the theory.

References

1. Böeseken, J., Vermaas, N. & Küchlin, A. Th.: La composition et l'acidité des acides boro-glycoliques. — Rec. trav. chim. 49: 711. 1930.
2. Burström, H.: Studies on growth and metabolism of roots. VI. The relative growth action of different isobutyric acid derivatives. — Physiol. Plant. 4: 470. 1951.
3. — Studies on growth and metabolism of roots. IX. Cell elongation and water absorbtion. — Physiol. Plant. 6: 262. 1953.
4. Caujolle, F. & Bergal, Georgette: Influence des acides borique et phénylborique sur la germination et le développement des plantules de quelques dicotylédones. — Compt. rend. Paris. 231: 1550. 1950.

5. — — Influence de l'acide α-naphtylborique sur la germination et le développement des plantules de quelques dicotylédones. — Compt. rend. Paris. 232: 109. 1951.

6. Fransson, P. & Ingestad, T.: The effect of an anti-auxin on the indole-acetic acid content in avena coleoptiles. — Physiol. Plant. 8: 336. 1955.

7. Gauch, H. G. & Dugger, Jr., W. M.: The role of boron in the translocation of sucrose. — Plant Physiol. 28: 457. 1953.

8. — — The physiological action of boron in higher plants: A review and interpretation. — Contribution No. 2581 of the Maryland Agricultural Experiment Station, Maryland. Dec. 1954.

9. Johnston, E. S. & Dore, W. H.: The influence of boron on the chemical composition and growth of tomato. — Plant Physiol. 4: 31. 1929.

10. Jönsson, Å.: Synthetic plant hormones. — Svensk kem. tidskr. 67: 166. 1955.

11. Torssell, K.: The chemistry of arylboric acids. I—V and VII. — Arkiv Kemi. 1957, in press.

12. Veldstra, H.: Ann. Rev. Plant Physiol. 4: 151. 1953.

Sulphite Oxidation by a Plant Mitochondrial System. Enzymic and Non-enzymic Oxidation

By

J. M. Tager and Niilo Rautanen [1]

Plant Physiological Research Institute, University of
Pretoria (South Africa)
(Received October 13, 1956)

It was recently shown that oat mitochondrial preparations bring about the enzymic oxidation of inorganic sulphite to sulphate and that maximal activity of the system is dependent on a supply of magnesium ions and cytochrome c (1). Among the more puzzling features of this system were the marked stimulation of the oxidation by azide at high concentrations, in contrast to a marked inhibition by cyanide, and a stimulation by adenosine monophosphate which appeared to be unrelated to the role of this nucleotide as a phosphate acceptor. Further study showed that stimulation was brought about by several other nitrogenous compounds and that under certain circumstances, the non-enzymic oxidation of sulphite could be similarly stimulated.

The results of experiments on the effect of various nitrogenous compounds, substrate concentration and other factors on both the enzymic and non-enzymic oxidation of sulphite are presented in this paper. Although we can offer no adequate explanations for most of these results, we feel that they may be of interest to other workers in this field.

Experimental

The material used, the procedure employed for isolating mitochondria from etiolated oat seedlings, the manometric method, the procedures for

determining nitrogen and sulphate and the reagents used were the same as those described earlier (1). Creatinine was determined by the method of Folin and Wu (2). All the manometric experiments were performed at 30° C.

Results

Effect of substrate concentration

In our earlier study of sulphite oxidation by oat mitochondria, a substrate concentration of 0.04 M was used in most of the experiments, since although the enzyme system did not appear to be saturated with substrate at this concentration, it was possible that higher sulphite concentrations might lead to complicating osmotic effects. The effect of higher substrate concentrations on the rate of sulphite oxidation was tested further, and the results of one such experiment are shown in Figure 1. Below a substrate concentration of 0.04 M, sulphite was not oxidized non-enzymically. Above this concentration, however, the non-enzymic oxidation of sulphite increased rapidly until, at a concentration of about 0.14 M, the oxygen uptake was the same in the absence as in the presence of mitochondria. In the presence of a mitochondrial suspension inactivated by heating at 90° C for 1 minute, the oxygen uptake was 0—10 µl per vessel per hour at sulphite concentrations up to 0.08 M.

Effect of nitrogenous compounds on the enzymic oxidation

The striking stimulation of sulphite oxidation by sodium azide or adenosine monophosphate observed previously by us led us to search for other compounds which might have a similar effect. A number of water-soluble compounds with structures analogous to azide or adenosine monophosphate were tested. It was found that creatinine, histamine, histidine and thiamine also stimulated oxidation (Figure 2). No oxygen uptake occurred in control vessels containing heat-inactivated mitochondria. Other compounds tested were nicotinic acid, nicotinamide, N-methyl nicotinamide, arginine, creatine and cyanamide. At a concentration of 0.02 M, cyanamide caused a 6 per cent stimulation of sulphite oxidation, while the other compounds caused an inhibition ranging from 12 to 32 per cent. Proline and hydroxyproline at 0.01 M caused inhibitions of 28 and 17 per cent respectively. With the exception of azide, all these stimulatory compounds have a ring structure and contain more than one nitrogen atom in the ring.

Since the stimulation of sulphite oxidation by azide, adenosine monophosphate, creatinine, histamine, histidine or thiamine was a function of the concentration of these substances and since maximal stimulation was obtained

Figure 1. *The effect of substrate concentration on the enzymic and non-enzymic oxidation of sulphite.* Reaction mixture contained: potassium phosphate buffer, pH 7.4, 0.02 M; MgCl$_2$, 0.002 M; cytochrome c, 3×10^{-5} M; sucrose 0.4 M; mitochondrial suspension, 0.5 ml. Final volume, 1 ml. Water substituted for the mitochondrial suspension in studying the non-enzymic oxidation.
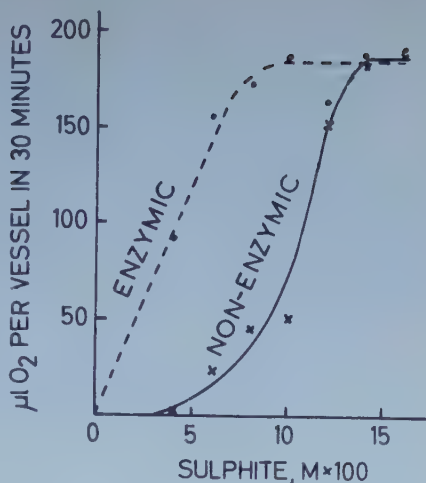
at concentrations of these compounds stoichiometrically similar to the concentration of sulphite used, it seemed possible that they might take part in the reaction in some way. Sulphite might for example react with these compounds to form products which would be oxidized in preference to sulphite itself. If the oxidized "complex" were sufficiently stable, the ratio of sulphate formed to oxygen taken up would decrease as compared with the ratio found in the absence of a stimulatory compound. The results of two representative experiments in which oxygen uptake and sulphate formation were determined in the presence and in the absence of stimulating compounds are presented in Table 1. Only in the case of thiamine was a slight decrease in the ratio of sulphate formed to oxygen taken up observed.

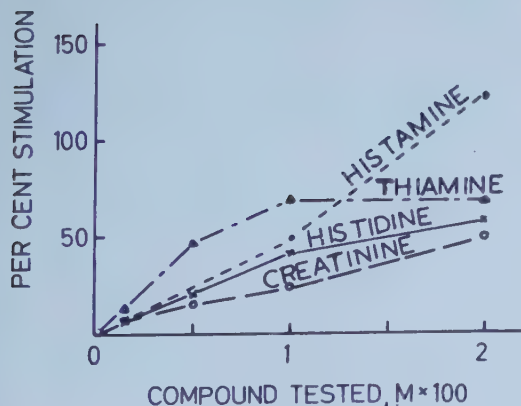If these compounds take part in the reaction during the oxidation of sul-

Figure 2. *The effect of certain nitrogenous compounds on the enzymic oxidation of sulphite.* Reaction mixture contained potassium phosphate buffer, pH 7.4, 0.02 M; MgCl$_2$, 0.002 M; cytochrome c, 3×10^{-5} M; sucrose, 0.4 M; sulphite, 0.04 M; mitochondrial suspension, 0.5 ml. Final volume, 1 ml. Oxygen uptake measured for 1 hour.

Table 1. *The effect of azide, histidine and thiamine on oxygen uptake and sulphate forma-tion during the enzymic oxidation of sulphite.* Conditions as for Figure 2.

Additions	Per vessel in 60 minutes		μmoles sulphate: μatoms 0
	μmoles sulphate formed	μatoms 0 absorbed	
None	11.8	10.2	1.1
Azide, 0.02 *M*	23.2	20.8	1.1
None	10.2	10.9	0.93
Histidine, 0.02 *M*	16.8	17.8	0.94
Thiamine, 0.02 *M*	16.0	19.4	0.83

phite, their concentration should decrease with time. When creatinine was tested as a stimulatory compound, its concentration was the same after sixty minutes as at zero time.

The possibility of these stimulatory compounds having an effect on the mitochondria themselves was investigated. Mitochondria isolated from 80 g of seedlings were divided into two equal portions, one of which was taken up in 15 ml 0.5 *M* sucrose and the other in 15 ml 0.5 *M* sucrose+0.04 *M* sodium azide. After 30 minutes at 2° C, the mitochondria from each portion were sedimented by centrifugation, washed once by suspension in 15 ml 0.5 *M* sucrose, resedimented and taken up in 0.5 *M* sucrose. The effect of azide on the rate of sulphite oxidation by these two preparations was deter-mined. Azide caused a stimulation of 141 per cent in the case of mitochondria pretreated with sucrose and of 165 per cent in the case of mitochondria pre-treated with sucrose-azide.

At this stage we became aware of the fact that the conditions of our experiments had fortuitously been chosen in such a way that the non-enzymic oxidation of sulphite was minimal. We found that sucrose, as well as other sugars, inhibited the autoxidation of sulphite (Table 2). Bigelow (3) had earlier found that a series of compounds. including hydroxyl com-

Table 2. *The effect of sugars on the non-enzymic oxidation of sulphite.* Reaction mixture contained potassium phosphate buffer, pH 7.4, 0.02 *M*; Sulphite, 0.04 *M*. Final volume. 1 ml.

Additions	μl O_2 per vessel in 30 minutes
None	242
Sucrose, 0.1 *M*	11
Sucrose, 0.2 *M*	0
None	206
Maltose, 0.1 *M*	40
Trehalose, 0.1 *M*	4
None	202
Glucose, 0.1 *M*	28

Table 3. *The effect of creatinine, azide and iodoacetate on the enzymic oxidation of sulphite.* Reaction mixture contained potassium phosphate buffer, pH 7.4, 0.04 M; MgCl$_2$, 0.002 M; cytochrome c, 3×10^{-5} M; sulphite, 0.02 M; ethylenediaminetetraacetic acid, 0.001 M; mitochondrial suspension, 0.5 ml. Final volume 1 ml.

Test No.	Enzyme	Additions	µl O$_2$ per vessel in 60 minutes	Stimulation per cent
1.	Untreated	None	75	28
		Creatinine, 0.02 M	96	
	Inactivated	None	9	
		Creatinine, 0.02 M	13	
2.	Untreated	None	119	59
		NaN$_3$, 0.02 M	190	
	Inactivated	None	35	
		NaN$_3$, 0.02 M	25	
3.[1]	Untreated	None	49	257
		NaN$_3$, 0.02 M	175	
	Inactivated	None	2	
		NaN$_3$, 0.02 M	10	
4.[1]	Untreated	None	76	112
		Iodoacetate, 0.02 M	161	
	Inactivated	None	3	
		Iodoacetate, 0.02 M	47	

[1] Azide and iodoacetate added from side arm at zero time.

pounds like mannitol, inhibited the autoxidation of sulphite. The mechanism of the inhibition appears to be unknown. We considered the possibility that compounds like sucrose might bind sulphite in a manner similar to the formation of borate complexes of sugars (see Pigman and Goepp, 4) and that the stimulation by compounds like azide of the enzymic oxidation of sulphite might be due to a reversal of this binding, perhaps by the compounds displacing sulphite in the hypothetical sucrose-sulphite complex. Accordingly, we tested the effect of azide and other compounds on the enzymic oxidation of sulphite in the absence of sucrose. Mitochondria were isolated in the standard way; but they were washed in 0.1 M potassium phosphate buffer, pH 7.4, instead of in 0.5 M sucrose, and taken up finally in the same buffer instead of in 0.5 M sucrose. Control vessels contained mitochondria inactivated by heating at 95° C for 1 minute. The results of these tests are presented in Table 3. The stimulatory effect of creatinine, azide and iodoacetate on the enzymic oxidation of sulphite was not dependent on the presence of sucrose.

Some factors affecting the non-enzymic oxidation of sulphite

It has been noted above that the non-enzymic oxidation of sulphite is inhibited by sugars. While studying the possible relationship between the

Table 4. *Some factors affecting the non-enzymic oxidation of sulphite.* Reaction mixture contained potassium phosphate buffer, pH 7.4, 0.02 M; and sucrose, 0.1 M, or ethylene-diaminetetraacetic acid, 2×10^{-5} M. The sulphite concentration was 0.08 M in the presence of sucrose and 0.04 M in the presence of ethylenediaminetetraacetic acid. Final volume, 1 ml.

Additions	Sucrose present		Ethylenediaminetetraacetic acid present	
	µl O_2 per vessel in 30 minutes	Stimulation or inhibition, per cent	µl O_2 per vessel in 30 minutes	Stimulation or inhibition, per cent
None	210		139	
MgCl₂, 0.002 M	214	+ 2	175	+ 26
Cytochrome c, 3×10^{-5} M	100	− 52	127	− 9
None	32		108	
Iodoacetate, 0.02 M	210	+556	293	+171
None	46		189	
NaCN, 10^{-4} M	211	+359	242	+ 28
NaCN, 10^{-3} M	153	+233	214	+ 13
NaCN, 10^{-2} M	10	− 78	137	− 27
None	212		189	
Thioglycollate, 0.001 M ..	38	− 82	87	− 54
Thioglycollate, 0.004 M ..	22	− 90	41	− 78
Thioglycollate, 0.02 M ...	18	− 91	17	− 91

presence of sucrose in the reaction mixture and the stimulatory effect of azide and other compounds on the enzymic oxidation of sulphite, we included some tests on the effect of these stimulatory compounds on the non-enzymic oxidation. When we found that these compounds did have a striking effect, we began to study the factors affecting the non-enzymic oxidation in greater detail. In all these tests, sulphite was added from the side-arm at zero time because the rate of oxidation was not linear but decreased rapidly with time. When sucrose was absent from the reaction mixture, ethylene-diaminetetraacetic acid was added in order to decrease the rate of sulphite oxidation.

In the presence of sucrose, the non-enzymic oxidation of sulphite was unaffected by magnesium ions, inhibited by cytochrome c and stimulated by iodoacetate, while in the absence of sucrose, magnesium ions stimulated, cytochrome c inhibited very slightly and iodoacetate stimulated (Table 4). At low concentrations, cyanide stimulated the non-enzymic oxidation of sulphite, whether sucrose was present or not; as the concentration of cyanide was increased, the stimulation became less, and cyanide was inhibitory at 0.01 M (Table 4). Thioglycollate markedly inhibited the non-enzymic oxidation of sulphite (Table 4).

The non-enzymic oxidation of sulphite was stimulated by azide both in the presence and in the absence of sucrose (Table 5). Creatinine, histidine

Table 5. *The effect of azide, creatinine, histidine and thiamine on the non-enzymic oxidation of sulphite.* Conditions as for Table 4, except that sulphite concentration was 0.04 *M*.

Additions	Sucrose present		Ethylenediaminetetraacetic acid present	
	µl O_2 per vessel in 30 minutes	Stimulation or inhibition, per cent	µl O_2 per vessel in 30 minutes	Stimulation or inhibition, per cent
None	58		132	
NaN$_3$, 0.002 *M*	50	− 14	180	+ 36
NaN$_3$, 0.01 *M*	74	+ 27	240	+ 82
NaN$_3$, 0.04 *M*	174	+ 200	315	+139
None	4		182	
Creatinine, 0.02 *M* ..	128	+3100	53	− 71
Histidine 0.02 *M*	57	+1325	15	− 92
Thiamine, 0.02 *M* ...	5	—	16	− 91

and thiamine inhibited the non-enzymic oxidation in the absence of sucrose; creatinine and histidine brought about a marked stimulation in the presence of sucrose while thiamine had no effect (Table 5).

Discussion

Several points of similarity were found in the enzymic and non-enzymic oxidation of sulphite. In both cases, there was a marked stimulation by azide and monoiodoacetate and an inhibition by thioglycollate. On the other hand, the enzymic oxidation was stimulated by cytochrome c and magnesium ions and inhibited by cyanide, while the non-enzymic oxidation was inhibited by cytochrome c, stimulated by magnesium ions only in the absence of sucrose, and stimulated by low concentrations of cyanide. Creatinine stimulated the enzymic oxidation in the presence and in the absence of sucrose.

The non-enzymic oxidation was stimulated by creatinine in the presence of sucrose and inhibited in the absence of sucrose.

Fridovich and Handler (5) found that xanthine oxidase treated with cyanide catalyzed the oxidation of sulphite in the presence of catalytic amounts of hypoxanthine. Below pH 7.6, cyanidetreated xanthine oxidase brought about the oxidation of sulphite in the absence of hypoxanthine. In the light of our observations the oxidation of sulphite at the lower pH values may perhaps be an effect of cyanide *per se* and independent of the presence of xanthine oxidase.

In contrast to the sulphite oxidase of mammals and microorganisms (Heimberg *et al.*, 6), the oat mitochondrial sulphite oxidase cannot use methylene blue as an electron acceptor. The rate of reduction of methylene

blue by sulphite was the same in the presence of native oat mitochondria as in the presence of heat-inactivated mitochondria. Hypoxanthine, considered by Fridovich and Handler (7) to be the coenzyme of purified dog liver sulphite oxidase, had no effect on the rate of methylene blue reduction by sulphite in the presence of oat mitochondria.

In our earlier study, we considered that the inhibition of the oat mitochondrial sulphite oxidation system by sulphydryl compounds might point to a requirement for disulphide linkages in the system. However, the non-enzymic oxidation is similarly inhibited by thioglycollate. Fridovich and Handler (5) suggest a role for disulphide linkages in dog liver sulphite oxidase, partly on the basis of an inhibition of their system by arsenite and p-chloromercuribenzoate.

The great susceptibility of inorganic sulphite to non enzymic oxidation r nders the study of the enzymic oxidation particularly difficult. We can at present offer no explanations for most of the observations reported in this paper.

Summary

Some factors affecting the oxidation of sulphite by a plant mitochondrial system and the non-enzymic oxidation of sulphite have been studied.

The enzymic oxidation of sulphite was stimulated by creatinine, histamine, histidine and thiamine.

The non-enzymic oxidation of sulphite was inhibited by sucrose and other sugars. In the presence of sucrose, the non-enzymic oxidation increased as the sulphite concentration increased.

The non-enzymic oxidation of sulphite was inhibited by creatinine, histidine and thiamine in the absence of sucrose, but stimulated by the first two compounds in the presence of sucrose.

A ide, iodoacetate and low concentrations of cyanide stimulated the non-enzymic oxidation of sulphite Higher concentrations of cyanide, and thioglycollate and cytochrome c caused an inhibition.

Magnesium ions stimulated the non-enzymic oxidation of sulphite in the absence of sucrose.

References

1. Tager, J. M. & Rautanen, N.: Sulphite oxidation by a plant mitochondrial system. — Biochem. Biophys. Acta 18: 111. 1955.
2. Folin, O. & Wu, H.: A system of blood analysis. — J. Biol. Chem. 38 81. 1919.

3. Bigelow, S. L.: Katalytische Wirkungen auf die Geschwindigkeit der Oxydation des Natriumsulfits durch den Sauerstoff der Luft. — Z. physik. Chem. 26: 493. 1898.

4. Pigman, W. W. & Goebb, Jr., R M.: Boric acid esters. — Chemistry of the Carbohydrates, Acad Press Inc. New York 1948 p. 183 and 253.

5. Fridovich, J. & Handler, P.: Sulphite oxidation by xanthine oxidase. — Inorganic Nitrogen Metabolism. Ed. by W. D. McElroy and B. Glass. The Johns Hopkins Press. Baltimore 1956, p. 539.

6. Heimberg, M., Fridovich, I. & Hand er, P.: The enzymatic oxidation of sulfite. — J. Biol Chem. 204: 913. 1953

7. Fridovich, I. & Handler, P.: Hypoxanthine, coenzyme of sulfite oxidase. — Federation Proc. 14: 214. 1955.

Some Notes on the Fluorescence Spectra of Plants *in vivo*

By

HEMMING I. VIRGIN

Dept. of Plant Biology, Carnegie Institution of Washington,
Stanford, Cal. U.S.A.[1]
(Received October 24, 1956)

In recent papers (French, 1955; French *et al.*, In Press) our present know-ledge of the fluorescence spectrophotometry of photosynthetic pigments has been reviewed. It is evident from these papers that fluorescence is a property of the chlorophyll pigments which can be used to solve many problems of a physical as well as a physiological nature. The most striking fact about fluorescence is the low concentration of pigments needed to give a measur-able intensity of the fluorescent light. It is therefore possible to make quan-titative estimations of pigment content in very small fragments of tissues (cf. Virgin, 1955).

The main part of the fluorescent light emitted by an illuminated green tissue derives from the chlorophyll pigments in the chloroplasts. It is there-fore quite natural that the spectral composition of the light should rather closely resemble that obtained from pigment extracts of the same tissue. There are, however, differences which are of great interest, as they might give a hint of the way in which light penetrates and is absorbed by tissues of varying anatomical construction.

In the present paper some fluorescence spectral curves of live plants are presented. The purpose is to show the great variability in the spectrum obtained. This variability can in most cases be explained by trivial phenomena such as light scattering and reabsorption within the tissues. The curves are only a few representative examples of many measurements but they show some of the different problems which can be encountered in the study of fluorescence *in vivo*.

[1] Present address: Botanical Laboratory, Lund, Sweden.

Method

The apparatus used for the measurements has been described earlier in other connections (French, 1955; French and Young, 1952).

The fluorescent light measured was emitted from the surface and first few underlying layers of the illuminated tissue. The incident light was the 436 mµ line isolated from a low pressure Hg-lamp by means of Corning filters No. 3389, 5113, and 4305. The fluorescent light was measured between 600 mµ and 770 mµ. Filters prevented any stray incident light within this wavelength range from reaching the sample. All measurements were performed after the attainment of the steady state in the fluorescence so that any change due to the so-called "Kautsky effect" (Kautsky and Franck, 1948; Wassink and Katz, 1939 a.o.) was eliminated.

Fluorescence of tissues with low pigment concentrations

When investigating green tissues from plants of different taxonomic relations for the emission of fluorescence under excitation by blue light, one finds a general similarity in the shape of the fluorescence spectra observed. It is a fluorescence spectrum of chlorophyll *a*, more or less distorted due to light scattering and reabsorption of the emitted fluorescent light. Only if other fluorescent material is present in comparable amounts will the spectrum obtained become influenced by this material. Examples of this are the spectra of red algae. As to the general shape of the fluorescence spectral curves of plants *in vivo*, see French (1955) for further references.

Figure 1 shows some fluorescence spectra from living tissues of various kinds. The material has been chosen so that distortion due to light scattering and reabsorption is low. (The tissues have few air spaces and a low chlorophyll content). It can be seen that the positions of the maxima vary within about 10 mµ. The reason for these variations is not known, but they may be due to variations in the protein-chlorophyll complex (cf. Rabinowitch, 1956; pp. 1847—1849). Ether extracts of the same tissues all show the same fluorescence spectrum with maxima at 668 mµ and around 723 mµ.

Influence of the thickness of chloroplast-containing layers

The effect of air-filled intercellular spaces on light-scattering in connection with fluorescence measurements *in vivo* was shown in a previous paper (Virgin, 1954). In normal terrestrial plants this effect is great but can be reduced by infiltration of the tissues. By using partly variegated plants it is possible to get an idea of the rôle of the thickness of the chloroplast-con-
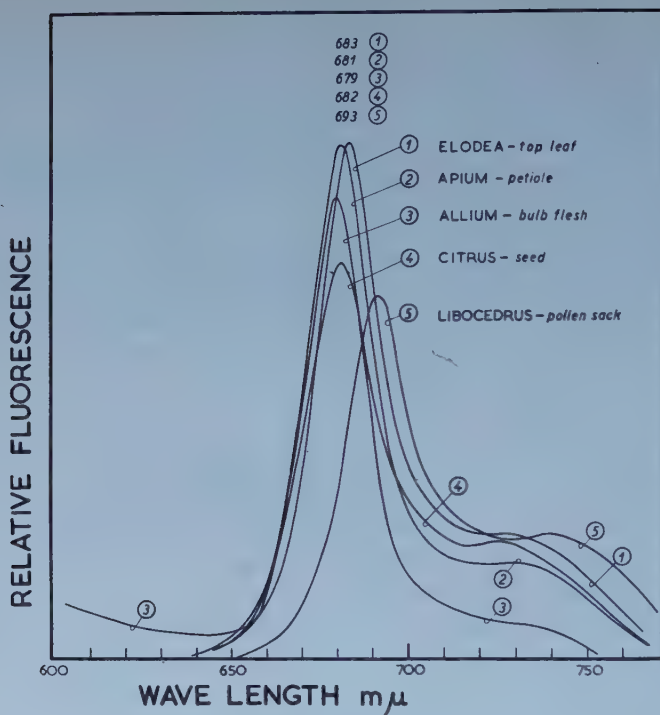
683 ①
681 ②
679 ③
682 ④
693 ⑤

① ELODEA – top leaf

② APIUM – petiole

③ ALLIUM – bulb flesh

④ CITRUS – seed

⑤ LIBOCEDRUS – pollen sack

RELATIVE FLUORESCENCE

WAVE LENGTH mμ

Figure 1. *Fluorescence spectra of some tissues containing low concentrations of chloro-phyll pigments.* Incident light and sensitivity of the recording instrument the same in all experiments except for No. 3 (Allium), where the sensitivity was three times higher than in the others. The fluorescence of the cellulose at wavelengths less than 650 mμ is clearly seen in this experiment; cf. Figure 4. The exact positions of the main maxima of the fluorescence spectra in this and the following Figures are given in mμ.

taining layers for the composition of the emitted light. In Figure 2 is shown the spectral composition of the fluorescent light emitted from different parts of variegated leaves of Hedera helix. The conclusion can be drawn from the curves that it is more or less insignificant for the spectral composition of the emitted light from a leaf containing only one layer of normal palisades whether or not the light passes a "chlorophyll-free" layer before it reaches the normal layer. These curves are similar and do not differ considerably from the spectrum obtained from a normal leaf. The so-called "chlorophyll-free" parts of the leaves do contain chlorophyll in small amounts as they show chlorophyll fluorescence (cf. also Figure 11. p. 62 in French, 1955). The spectral composition of the light emitted from a "chlorophyll-free" layer, however, is quite different and resembles very much the spectrum
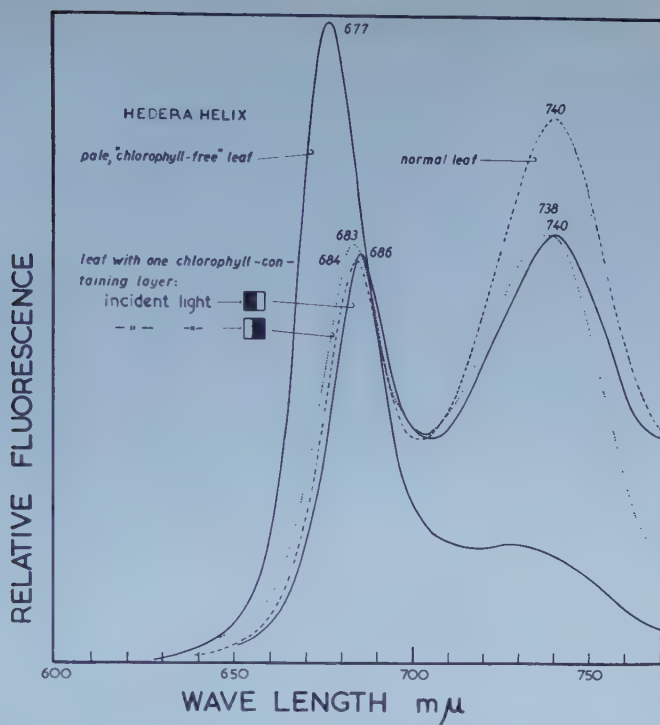
Figure 2. *Fluorescence spectra of different parts of a variegated leaf of Hedera helix.* Incident light and sensitivity of the recording instrument the same in all experiments. Chlorophyll-containing layer is indicated black in the Figure.

which will be obtained from a weak extract of the pigments. This shows the great reabsorption of fluorescent light by the chlorophylls in normal plants, a fact clearly shown by French and Young (1952) and which also finds expression in the shift of the maximum in the spectrum of the variegated form at around 677 mμ to around 685 mμ for the forms containing more or less normal chlorophyll containing layers.

The fluorescence spectrum obtained is presumably that of chlorophyll *a* due to the practically complete energy transfer from chlorophyll *b* to chlorophyll *a* in an illuminated green plant (Duysens, 1952). The concentration of pigments in "chlorophyll-free" parts, however, is low and the pigments are hardly detectable by absorption spectrophotometry unless extracts are made from many leaves. Thanks to the very low concentration the distortion due to reabsorption is small or absent and the spectrum shows great similarity to that of pure pigments in solution (French *et al.*, In Press).

Interference of other fluorescing material

In cases when several fluorescing substances are present at the same time in the tissue the spectrum obtained will become very confusing particularly when such phenomena as light scattering and reabsorption are predominant. In marine algae which contain at least three differently fluorescing substances (phycoerythrin, phycocyanin and chlorophyll *a*) the resultant fluorescence spectrum closely resembles the sum of those of the single pigments (French and Young, 1952). In this case — unicellular water organisms — no air-filled spaces interfere with the measurements. Also the reabsorption of fluorescent light due to high concentrations of pigments plays a minor rôle since fluorescence maxima and absorption maxima are well apart. Estimations of the absolute amounts of a single pigment can thus in this case be made by adding the curves of the components in suitable proportions to match the experimentally obtained curve. In some terrestrial plants, however, light scattering and reabsorption can become so strong that a resolution of the experimentally obtained curves into the fluorescence spectra of individual pigments is hardly possible. In order to show this a series of spectral curves are presented in Figure 3, representing the fluorescence from various layers of the inner seed coats of "Hubbard's squash" (Cucurbitaceae). The inner seed coats of this plant are particularly rich in protochlorophyll in a photochemically inactive form, *i.e.* it does not transform into chlorophyll *a* under the influence of light. Figure 3 shows the difference in fluorescence spectrum when the outer layers of the seed coat are excited by the incident light and when the fluorescence is excited in the inner layers. Disregarding minor differences, three maxima can be seen in the spectrum, one very distinct at around 710 mμ and two others at 630 mμ and 660 mμ respectively. The explanation for the formation of these three peaks is given by French (1955) taking into account the fact that protochlorophyll according to Smith and Benitez (1953) exists in two forms — one with an absorption peak at 635 mμ, the other at about 650 mμ. These two peaks should thus give fluorescence bands some wavelengths shifted towards red. These bands are, however, strongly reabsorbed in comparison to a minor long wavelength band around 710 mμ. A most striking fact, shown in Figure 3, is that two of the peaks are hardly noticeable unless the tissues are treated so that the light scattering is diminished. Only by pressing the tissues or infiltrating them so that the intercellular spaces disappear it is possible to obtain clearly the peak at around 630 mμ belonging to the more stable form of the two protochlorophylls. By comparing this Figure 3 with Figure 14 in the work by French (1955) it is quite obvious that the shape of the fluorescence spectrum is unpredictable when the material used has a
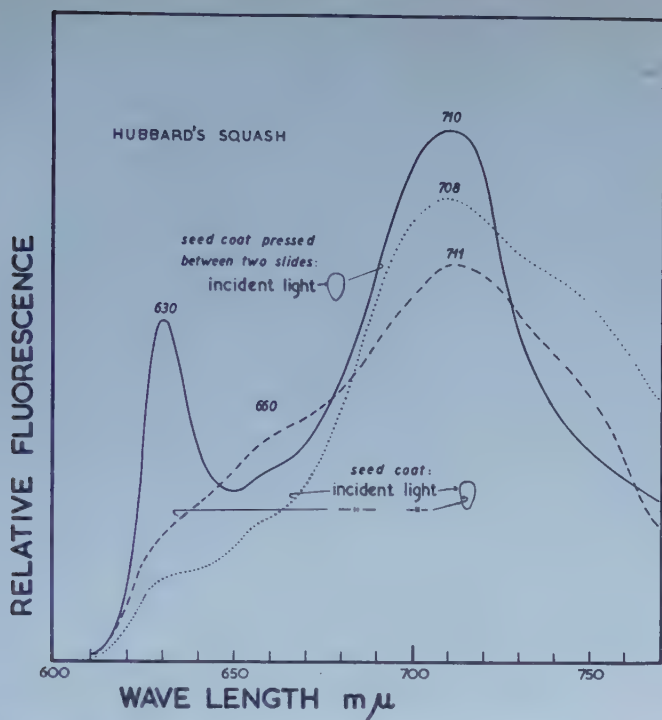
Figure 3. *Fluorescence spectra of the inner seed coat of Hubbard's squash.* Incident light and sensitivity of the recording instrument the same in all experiments.

rather complicated "optical structure". Any change in "the optical structure" of the tissue will bring about another variation in the emitted fluorescent light. Due to the complex nature of the tissue *in vivo* it is with our present means not possible to make corrections for these distortions except for certain material (cf. Virgin, 1955).

When the chloroplast pigments occur in very small amounts in the plant, their presence *in vivo* can be detected only by means of spectrofluometry. In this case the presence of other fluorescing materials will become increasingly disturbing as the intensity of this fluorescence approaches the intensity of fluorescence of the chloroplast pigments. Normally the concentration of the fluorescing pigments in the plants, mainly consisting of chlorophylls, reach such a level that one does not have to pay any attention to extraneous fluorescence, particularly since very few substances except the chlorophylls show red fluorescence (Goodwin, 1953). In Figure 4 are given examples showing the interference of cellulose fluorescence. Such an interference will become actual when working with woody tissues but is detect-
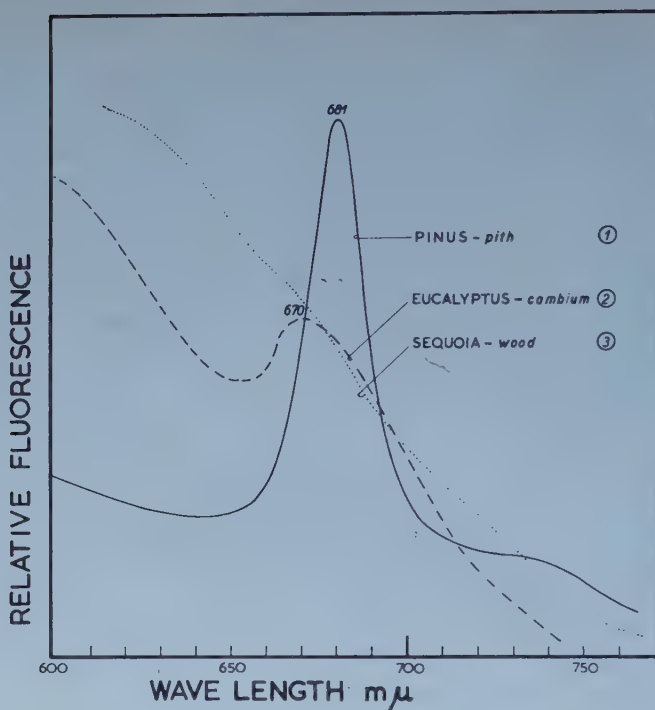
Figure 4. *Fluorescence spectra of some woody tissues containing very low concentrations of chlorophylls.* Incident light the same in all experiments. Sensitivity of the recording instrument in ratio $1:2:3$ for the experiments No. 1, 2, and 3 respectively.

able even in fleshy tissues if working with high sensitivity of the measuring instruments. The fluorescence emitted by cellulose and its derivatives has its energy maximum in the blue part of the visible spectrum. This implies that the spectral curve rises rather steeply towards shorter wave-lengths. The limit of the fluorescence in red is around 760 mμ. The examples presented in the Figure show clearly the interference of the cellulose fluorescence with the determination of the fluorescence spectrum of pigments that are present in small amounts. The Figure also shows that one has to take into account very small amounts of chlorophyll pigments in tissues which are considered to be chlorophyll-free.

Summary

Fluorescence-spectrum curves of living plants are presented which show the effect of different factors on the composition of the fluorescent light. In

particular the effect of light scattering and reabsorption is illustrated by several examples. The interference by other simultaneously present fluorescing material is illustrated with material containing low concentration of chlorophyll. In such cases the fluorescence of the cellulose in the cell walls will become increasingly evident.

The experiments presented in this paper were performed when the author was a fellow at the Carnegie Institution of Washington, and he wants to express his thanks to Drs C. S. French and James H. C. Smith for help and encouragement in the work.

References

Duysens, L. N. M.: The transfer of excitation energy in photosynthesis. — Thesis. Utrecht 1952.

French, C. Stacy: Fluorescence spectrophotometry of photosynthetic pigments. — In: The Luminescence of Biological Systems. Ed.: Frank H. Johnsson. Washington D. C. 1955.

— Smith, James H. C., Virgin, Hemming I. & Airth, Robert L.: Fluorescence-spectrum curves of chlorophylls, pheophytins, phycoerythrins, phycocyanins and hypericin. — Plant Physiol. In Press.

— & Young, V. K.: The fluorescence spectra of red algae and the transfer of energy from phycoerythrin to phycocyanin and chlorophyll. — J. Gen. Physiol. 35: 873. 1952.

Goodwin, Richard H.: Fluorescent substances in plants. — Ann. Rev. Plant Physiol. 4: 283. 1953.

Kautsky, H. & Franck, U.: Fluoreszenzanalyse des Lichtenergiewechsels der grünen Pflanze. Naturwissenschaften 38: 43. 1948. — Ibid. 35: 74. 1948.

Rabinowitch, Eugene I.: Photosynthesis and Related Processes. Vol. 2; part 2. — New York 1956.

Smith, James H. C. & Benitez, Allen: The protochlorophyll — chlorophyll transformation: the nature of protochlorophyll in leaves. — Carnegie Inst. of Wash. Year Book 52: 148. 1953.

Virgin, Hemming I.: The distortion of fluorescence spectra in leaves and its reduction by infiltration. — Physiol. Plant. 7: 560. 1954.

— The conversion of protochlorophyll to chlorophyll a in continuous and intermittent light. — Physiol. Plant. 8: 589. 1955.

Wassink, E. C. & Katz, E.: The initial changes of chlorophyll fluorescence in Chlorella. — Enzymologia 6: 145. 1939.

Temperature and Ròot Cell Elongation

By

Hans Burström

Botanical Laboratory, Lund
(Received October 25, 1956)

In a series of papers a theory of root cell elongation has been outlined, based on the assumption that the elongation takes place in two steps. The first phase should involve a loosening of the cell wall and a plastic stretching, the second phase an active wall formation by intussusception of new micro-fibrils (Burström 1955). It has been concluded that the latter phase should particularly require Ca-ions and the first phase should be sensitive to an inhibition by coumarin. These two natural growth regulators could be used as tools for distinguishing between the two growth phases. As to the point of action of IAA, it has been suggested that it promotes the first phase and inhibits the second one (Burström 1942 b).

In order to substantiate this theory experiments have been carried out on the effect of different temperatures on the root cell elongation and on the effect of the mentioned growth regulators.

The assumed two mechanisms of elongation should be of such a different nature that it is possible they possess different temperature coefficients. In such a case also the activities of the growth regulators should show different temperature dependence. This assumption has been verified in the following experiments on the course of the normal root cell elongation and the actions of Ca-ions and coumarin at different temperatures.

Some particulars concerning the relation between temperature and normal course of elongation of wheat roots have been given in a previous paper (Burström 1942 a). The total growth in length of the roots has an optimum around 20°C, decreasing towards 10°C and 30°C in accordance with findings by Richardson (1956). Conflicting statements in the earlier literature

are hardly surprising. If the wheat root growth is analyzed, it is found that the real rate of stretching of the individual cells or segments of the root increases rapidly up to 30°C but the grand period of elongation is very much shortened with increasing temperature, so that the final length attained by the cells decreases. The cell elongation is normally cut off by some unknown factor, which might be identical with IAA. In this way the final length of the cells and the root may decrease with increasing temperature, in spite of an increasing rate of stretching, owing to a shortened duration of the period of elongation of the individual cells. On the other hand, the rate of cell formation and number of cells at any moment in the progress of elongation may increase. For these reasons it is necessary to specify how root elongation is measured. In the present investigation the determination is based on *the final length attained by the individual cells of the root epidermis.*

Methods

The methods employed have been essentially the same as in previous studies (Burström 1953, 1954 a) and a detailed description is unnecessary here. Plant material was Eroica wheat studied during the heterotrophic seedling stage, grown in darkness on a complete nutrient solution of pH around 6.5 at 10°, 20°, and 30° C. The cell elongation was recorded microscopically; the error of the individual average values amounted to ±3 per cent. Each experiment was run in duplicate and repeated up to six times. The cell wall properties were studied by growing the seedlings with additions of 0.2—0.3 M mannitol to the nutrient solutions; for the computations of cell wall elasticity and plasticity see table 1. For this purpose the cultures were kept aseptic from the start and all precautions were taken to minimize the danger of infection during the experimental time. Not all manipulations, especially not the changing of the solutions, usually carried out every second day, could be done under wholly sterile conditions. Tests markedly infected were discarded. In order to insure uniformity of the experiments even those run without mannitol were handled with aseptic technique.

Temperature and cell wall properties

The experiments on the relation between temperature and the tensibility of the cell walls have been condensed in Table 1, showing average values for cell lengths under different conditions. The duration of the treatments differed, 2 days at 20° and 30°C, 4 days at 10°C, but this does not jeopardize a comparison; in all instances sections for the cell measurements were cut out immediately behind the zone of elongation, *i.e.*, cells were compared which had fully developed and just completed their elongation under the different conditions.

Table 1. *The effect of the temperature on the cell wall properties.* Plants grown without mannitol and with mannitol 0.2—0.3 M; averages of 5 to 6 independent experimental series, each with duplicates and mean errors of the cell lengths of ± 3 per cent. Cells plasmolyzed in 0.3 M mannitol.

Tempera- ture °C	Mannitol	Cell lengt μ			Elasti- city μ	Plasti- city μ
		Normally	Water saturated	Plasmolyzed		
10	—	262	✕	224	38	✕
	+	193	220.	212	8	19
20	—	231	✕	197	34	✕
	+	212	248	234	14	22
30	—	210	✕	191	19	✕
	+	196	209	193	16	0

A source of error not accounted for in these tests is the direct effect of the temperature on the viscosity of water, which increases considerably with decreasing temperature, and on the corresponding physical properties of the cell colloidal structures, including the micellary frame-work of the cell walls. The microscopic determinations were carried out at room temperature (20—22°) for all treatments and are thus in one way mutually comparable. However, this means that the cell properties recorded do not necessarily tally with those prevailing at the respective test temperatures. The elastic tensibility at 10°C ought to be lower than the value measured on the 10°-roots. On the other hand, the property of interest seems to be differences due to the structures built up at the different temperatures, not differences caused by the influence of temperature on this structure. In any case the results should be interpreted with some caution.

However, three points of importance are revealed in the experiments. Firstly, that in normal roots the final cell length decreases with increasing temperature, which was already known. Secondly, that the normal wall elasticity decreases with increasing temperature, and, thirdly, that the cell wall plasticity disappears at 30°C. The last-mentioned point should be especially emphasized. It is of particular interest and can hardly be explained as due to technical errors, whereas the decreasing elasticity may be at least partly influenced by the mentioned shift in temperature during the readings, which required between one and two hours. This renders the interpretation of the elasticity figures difficult.

The conditions at the supraoptimal temperature of 30° resemble in several respects those caused by an increased IAA level. This leads to a reduction in the final cell length by shortening the duration of the elongation and a shift from a plastic to an elastic tensibility of the cell walls (Burström 1942 b, 1954 b). The similarity with the high temperature effect is striking.

Temperature and calcium requirement

Under optimal conditions, around 20°C, maximal elongation is obtained with 10^{-4} M in the nutrient solution (Burström 1954 a). There is, however, a time factor in the appearance of calcium deficiency at lower concentrations; this will be dealt with in a forthcoming paper. It suffices to mention in this connexion that with Ca 10^{-7} M at 20°C the cell elongation lags behind significantly from the second day onwards. The two concentrations 10^{-7} and 10^{-4} M have been chosen in the experiments recorded in Figure 1.

Again very different durations of the experiments had to be chosen, in order to eliminate the unequal rates of elongation and general development of the seedlings. On the other hand, the experimental time could not be extended too much. The seedlings were grown in darkness developing heterotrophically. It can be mentioned as an indication of the rate of development and duration of the heterotrophic phase that the N-content of the seeds only permit a normal growth at 20°C for around four days (Bosemark 1954), and the seeds are exhausted after 7 days. After four days at 20°C the roots had attained a length in one experiment of 88 mm and formed longitudinally about 250 new cells; the dry weight of the seeds had decreased from 0.532 to 0.285 g per 16 seeds. At 10°C about the same root length (83 mm) was attained after 8 days, the cell number amounted to about 190, and the dry weight of the remnants of the seeds to 0.247 g. The plant dry weight per 16 seedlings was in the former case 0.204 and in the latter instance 0.230 g. These two stages of development ought to be approximately comparable.

Nevertheless, whereas the roots at 20°C exhibited an increasing Ca-deficiency, those at 10°C did not show the slightest response to Ca at this stage. A difference between the two calcium levels did not appear until towards the eleventh day, when the seeds were nearly depleted of nutrients and the growth began to decline rapidly. At 30°C the low-Ca roots lagged behind already on the first day with a total root length of not more than 30 mm.

Besides the rapid onset of calcium deficiency and decreasing cell elongation at 30°C the noticeable result is *the lack of calcium effect at 10°C.* The roots elongate at an optimal rate for the whole heterotrophic period of about 10 days at such a low calcium concentrations as 10^{-7} M or 0.004 p.p.m. The recorded general data of the developmental stage exclude the possibility that it is only due to a more economic use of the available quantity of calcium at the lower rates of growth. The conclusion must be drawn that for the same elongation accomplished the requirement of calcium is very much smaller — at least 1 : 1000 — at 10°C than at 20°C.

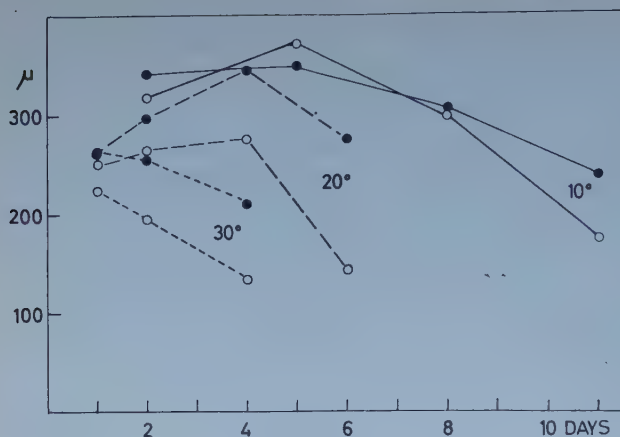This result can be described in two exactly opposite ways: *either* that

Figure 1. *The influence of calcium on the root cell elongation at different temperatures.* ○ Ca 10^{-7} M, ● Ca 10^{-4} M, ——— 10° C, — — — 20° C, - - - - - 30° C.

at 10°C the roots for maximal elongation are content with a low amount of Ca, *i.e.,* calcium is effectively utilized, *or* that the roots are unable to utilize more than a small amount for the elongation, which means that the utilization of calcium is impaired. No definite decision can be made because the changes in the roots due to the temperature are so diverse that a direct comparison between the growth responses at low and high temperature is impossible. With regard to the high level of elongation at 10° and the decline with time at higher temperatures it seems justified for the following discussion to assume that *at high temperature some factor retards the elongation and decreases the utilization of calcium.*

Temperature and coumarin inhibition

The corresponding experiments with additions of coumarin are summarized in Table 2. The time factor has not been considered in this table. The reason is that extensive series of experiments with flowing nutrient solutions particularly devoted to the study of the time element have revealed that the coumarin inhibition changes with the time, but in a manner, which at present is not clearly understood. A transient initial effect is shown in Figure 2. The data of table 2 referring to the first three days are simple and clear-cut. The figures denoting cell lengths show *a coumarin inhibition decreasing regularly with increasing temperature.* A 50 per cent reduction in cell length is achieved at the following concentrations: —

10°	20°	30° C
$1.1 \cdot 10^{-5}$	$2.4 \cdot 10^{-5}$	$4.2 \cdot 10^{-5}$.

Table 2. *The influence of coumarin on the cell elongation at different temperatures.* Cell lengths in μ. For mean errors see table 1.

Coumarin M	Temperature °C		
	10	20	30
0	277	246	216
10^{-5}	144	198	229
$3 \cdot 10^{-5}$	51	99	130
10^{-4}	0	46	98

The roots are less sensitive at 30°C in spite of the probably more rapid absorption; a destruction of coumarin may occur, but the solutions were supplied with a large excess. As is seen from the table coumarin actually reverses the temperature curve, a fact which should be borne in mind because coumarins are naturally occurring compounds. A more curious consequence is that since the three temperature curves cross each other, a concentration of coumarin can be found — approximately $4 \cdot 10^{-6}$ M — at which the root cell elongation turns superficially temperature-independent. However, this may be only seemingly, because the roots are certainly not identically constructed at different temperatures.

A careful study was made of the microscopic appearance of the roots treated with 10^{-5} M coumarin at 10°C. They exhibited sufficiently low rate of elongation and strong inhibition to reveal some interesting details.

If roots are transferred to any test solution from the moist filter paper on which the seeds have germinated their elongation is immediately checked. This has been observed repeatedly. The reason is that cells in progress of rapid elongation are extremely delicate and do not endure a sudden change in the environment. The cells of the zone of elongation at the start of an experiment are fixed in a half-developed state and are easily recognized microscopically (cf. Figure 2). There is a sharp line of demarcation between this part and the cells growing out in the new solution, usually reacting at once to the new environment. This is the case with, *e.g.*, an IAA inhibition.

With coumarin there is a peculiar transition zone between the old root and the part developing under the influence of the inhibitor (Figure 2). The five segments recognized are the following ones, from the base to the tip: —

A. The fully developed root at the transfer to the coumarin solution; cell length about 250 μ.

B. The zone of elongation at the commencement of the experiment; the cells have been fixed at different stages, which is most easily seen from the towards the tip regularly decreasing root hair lengths. This is a normal phenomenon.

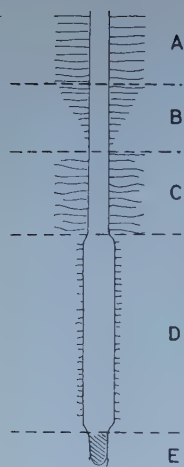*C.* A transition zone; the cells are practically normal in appearance, although

Figure 2. *Schematic structure of roots after three days in coumarin* 10^{-5} *M at 10° C*. Length scale founded on measurements on 12 roots of average length; width exaggerated. *Zones*: *A* Initial part at the commencement of the treatment; cell lengths about 250 µ. *B* Zone of elongation at the commencement. *C* Transition zone; 15 cells of an average length of 232 µ; general appearance almost normal. *D* Coumarin-inhibited part; cells averaging 144 µ, slightly swollen with short root hairs. *E* Normal meristem.

the root hairs are slightly irregular; average cell length 232 µ. This part comprising 15 cells in the longitudinal direction has grown out after the transfer to the coumarin solution; the time of its development has been estimated to 16 hrs.

D. Sharply set off from *C*, revealing coumarin inhibition; cells averaging 144 µ, slightly swollen, of a peculiar glossy and semi-transparent appearance; root hairs short, coarse, not of the auxin type. A slight swelling of the root in coumarin has also been observed by San Antonio (1956). This growth condition goes on rather unchanged, in the instance depicted for 56 hrs.

E. The meristem; morphologically normal and with rate of cell multiplication undisturbed.

The transition zone (*C*) is specific for coumarin treatment. It has developed in the presence of coumarin from a restricted number of cells apically of those visibly in progress of elongation at the application of coumarin. They grow out practically normally. One explanation would be a lag in the action of coumarin owing to the absorption of the compound. This possibility is hardly probable for two reasons. Firstly, because the inhibition would then reasonably develop gradually and there would be no sharp border line between zones *C* and *D*. Secondly, because the time 16 hours seems to be far too long for the absorption of an easily permeating compound in the young cells of the root, even at 10° C. The phenomenon is interpreted as follows.

This zone comprises the cells which on the application of coumarin had commenced their elongation without having reached the state of rapid elongation and root hair initiation, but which had passed the preliminaries of the elongation. The number of cells tallies with the shape and extension of the

elongation curve. Once this preliminary stage has been passed the cells are apparently insensitive to coumarin and continue elongating until the normal, final cell length is attained, in spite of the presence of coumarin in the surrounding solution. The conclusion would then be that the first part of the elongation, which corresponds to what in the introduction was called the first phase, is sensitive to coumarin, and that the inhibition is restricted to this very part. This is a histological confirmation of the conclusion that the effect of coumarin is limited to the first phase of the elongation, which has been deduced from the influence on the cell wall tensility (Burström 1954 b); this is assumed to be built up during the first phase, and it is destroyed by coumarin.

Comments

No temperature experiments have been carried out with additions of IAA in connexion with the recorded calcium and coumarin trials. The reason is the disturbing time factor in the IAA action, appearing as an adaptation. This necessitates at least tests with continuously flowing solutions, not employed in the temperature series. Since the real nature of the adaptation is unknown the interpretation of temperature experiments with IAA is hazardous, particularly if it involves an enzymatic destruction of IAA (cf. Galston and Dahlberg 1954). This adaptation will be treated in a separate communication.

The present investigation has shown that with increasing temperature the cell wall plasticity disappears but the elasticity remains; the sensitivity to coumarin decreases and the calcium requirement increases. It has furthermore been shown earlier (Burström 1942 b) that the duration of the elongation is shortened. The temperature effect on the roots and their metabolism is so complex that a definite interpretation of the results is difficult, but some points should be emphasized.

The study was started from the presumption that the first and second phases of the elongation were differently sensitive to a change in temperature. it would *a priori* be assumed that the active cell wall synthesis should have a higher temperature coefficient than passive stretching. The results could be interpreted accordingly, but for the fact that the final outcome of the elongation at high temperature consists in a reduced cell length. This means that an inhibiting factor with a positive temperature dependence is at work.

Another explanation is also possible. Roots at $30°$ C resemble those with a high IAA supply: the shortened duration of the elongation with ensuing reduction in cell length and the disappearing plasticity follow an application of IAA (Burström 1942 b, 1954 b). Furthermore an auxin addition decreases

the utilization of calcium (Burström 1954 b, 1955), which has been assumed to occur at high temperature. It is true that the actual data on the relation between 1-NAA and Ca (Burström *loci cit.*) do not agree well with the temperature experiments, but it must be recalled that a direct comparison is impossible and that an external supply of an auxin raises the internal level more than normally occurring supply. In principle the calcium responses at high temperature and high auxin level are of the same type.

As to coumarin, it may be possible that an increased destruction at high temperature must be allowed for. On the other hand, it is impossible to predict the interaction between coumarin and IAA, but unpublished results indicate a reduced coumarin inhibition in IAA-treated and adapted roots; data in the literature point in the same direction.

Summarizing, *an increase in temperature might cause primarily only an increased level of IAA,* which in its turn according to experimental evidence would lead to (i) shortened duration of the elongation and a reduced cell length, (ii) reduction in cell wall plasticity, (iii) increased calcium requirement and perhaps (iv) decreased sensitivity to coumarin.

Such an interpretation tallies with all known facts. The important point is that it has been possible by changing the temperature to separate the coumarin and calcium actions and change the wall properties in a manner consistent with the formulated general principles of the cell elongation mechanism.

The only information found in the literature on the auxin content of plants grown at different temperatures is reported by Gustafson (1946). He observed in most cases a lower content of auxin determined by the Avena curvature test at lower temperatures in different plants, but exceptions occurred, and the control of other environmental conditions was not quite satisfactory. Even the background of such IAA determinations is controversial. Working with a more accurate analytical procedure and additions of antiauxins Fransson and Ingestad (1955) conclude that the easily extractable, free IAA is not the fraction active in the elongation, but this is disputed by Audus and Thresh (1956) on the ground of experiments with other inhibitors.

The presumed second growth phase should probably correspond to the second phase described by Scott *et al.* (1956) on account of electron microscope studies of growing cell walls. Our first phase may correspond to theirs, but may also be regarded as a preparatory change in the wall, not necessarily showing up under the microscope but indispendable for the incorporation of new microfibrils, taking place both during the first and second phases according to the classification of Scott *et al.*

The location in the first growth phase of the coumarin action seems to be fairly well established also histologically, and it is distinctly separated from

the calcium effect, and histologically also from the inhibition by IAA (cf. Burström and Hansen 1956). Ca and IAA are, however, mutually interrelated, IAA decreasing and Ca increasing the second growth phase. Ca-ions do not act as ideal auxin-antagonists, which has previously been emphasized with no explanation offered (Burström 1952); but, nevertheless, auxin prevents the utilization of Ca. This interrelation can be seen in a new light after the suggestion of Heath and Clark (1956) that IAA acts by virtue of its chelating capacity. The intricate question then arises, what cation is chelated and whether the cation is the real growth regulator, IAA, being degraded to a regulator of the cation supply. As is always the case with chelates, a chelation can both increase and decrease the cation supply, according to the relative proportions between chelating agent and metal, and the complex stability. One excellent example is given by Gäumann (1956).

As far as root elongation is concerned only Ca-ions are at present known as specific regulators, and if the chelate principle holds true, IAA might act by binding Ca. All temperature results could be well interpreted on such a basis, but all chemical evidences are lacking. The remarkably low calcium optimum at $10°$ C, which lies at or below 10^{-7} M, would then imply that a higher calcium supply would not be required or utilized for the elongation *per se*, only as an antidote of IAA at higher temperatures. Such a principle could explain the remarkable difference in calcium requirement at high and low temperature.

Summary

The temperature effect on root cell elongation, cell wall properties and the growth-regulating actions of Ca and coumarin have been studied. With increasing temperature the final cell length decreases owing to a shortened duration of the elongation, the plasticity of the cell wall disappears, the requirement of calcium increases, and the roots become less sensitive to coumarin inhibition.

At $10°$ C the roots exhibit optimal elongation at a calcium concentration of 10^{-7} M (0.004 p.p.m.). It is shown histologically that the strong coumaring inhibition at this temperature is restricted to the first part of the elongation.

Increasing temperature resembles an increase in the level of active IAA, which tentatively can explain all changes observed. The nature of the interaction between IAA and Ca has been discussed.

References

Audus, L. J. & Thresh, R. The effect of synthetic growth regulator trea'ments on the levels of free endogenous growth-substances in plants. — Ann. Bot. 20: 439. 1956.

Bosemark, N. O.: The influence of nitrogen on root development. — Physiol. Plant. 7: 497. 1954.

Burström, H.: Die osmotischen Verhältnisse während des Streckungswachstums der Wurzel. — Ann. Agr. Coll. Sweden 10: 1. 1942 a.

— The influence of heteroauxin on cell growth and root development. — Ibid. 10: 209. 1942 b.

— Studies on growth and metabolism of roots IX. Cell elongation and water absorption. — Physiol. Plant. 6: 262. 1953.

— Studies on growth and metabolism of roots X. Investigations of the calcium effect. — Ibid. 7: 332. 1954 a.

— Studies on growth and metabolism of roots XI. The influence of auxin and coumarin derivatives on the cell wall. — Ibid. 7: 548. 1954 b

— Zur Wirkungsweise chemischer Regulatoren des Wurzelwachstums. — Bot. Not. (Lund) 108: 400. 1955.

— & Hansen. B. A. M: Root growth effects of indan, indene, and thionaphthene derivatives. — Physiol. Plant. 9: 502. 1956

Fransson, P. & Ingestad, T: The effect of an antiauxin on the indoleacetic acid content in Avena coleoptiles. — Ibid. 8: 336. 1955

Galston, A. W. & Dahlberg, L. Y.: The adaptive formation and physiological significance of indoleacetic acid oxidase. — Amer. J. Bot. 41: 373. 1954.

Gäumann, E. & Naef Roth, St.: Über die chelierende Wirkung einiger Welketoxine. IV. Die Verschiebungen der Toxizität durch steigende Absättigung mit verschiedenen Schwermetallen. — Phytopathol. Zeitschr. 25: 418. 1956.

Gustafson, F. G.: Influence of external and internal factors on growth hormone in green plants. — Plant Physiol. 21: 49. 1946

Heath, O. V. S. & Clark, J. E.: Chelating agents as plant growth substances. — Nature 177: 1118. 1956. — 178. 600. 1956.

Richardson, S. D.: Studies of root growth in Acer saccharinum L. IV. The effect of differential shoot and root temperature on root growth. — K. Ned. Akad Wet. 59: 428. 1956.

San Antonio, J. P.: The role of coumarin in the growth of roots of Melilotus alba. — Bot. Gaz. 114: 95. 1952.

Scott, F. M., Hamner, K. C., Baker, E. & Bowler, E.: Electron microscope studies of cell wall growth in the onion root. — Amer J Bot. 43: 313. 1956.

Differences in the Auxin Content of Wheat Roots Caused by Changes in the pH Value of the Nutrient Solution

By

Henry Rufelt and Pär Fransson

Botanical Laboratory, Lund
(Received November 8, 1956)

This is a short communication concerning experiments which have been performed with the aim of testing whether an increase of the pH value in the root medium from pH 5 to higher values is followed by a change in the amount of the short time extractable indole-3-acetic acid (IAA) in wheat roots.

The wheat plants were cultivated in a nutrient solution of the following composition: $Ca(NO_3)_2$ 10^{-3} M, KNO_3 10^{-3} M, $MgSO_4$ $5 \cdot 10^{-4}$ M, Fe^{3+}-citrate 10^{-5} M and KH_2PO_4 10^{-5} M. The pH of this solution was about 5. In the experiments the pH value was changed by means of a phosphate buffer. The plants were cultivated on a clot net stretched tightly over a shallow dish containing the nutrient solution. The dishes were placed at constant temperature ($22°C$) and the plants were illuminated with 100 W fluorescent lamps, daylight type. The nutrient solution was renewed every morning and after three days the plants were used for the experiments.

When an experiment was started, treatments were carried out in the following way. In one dish the solution was renewed (pH 5). In other dishes the solutions were changed to solutions with higher pH values (6, 6.5 and 7.5). In the solution of pH 7.5 there was always a precipitate of Ca-phosphate, which must have a buffering effect. In the experiment with 24 hours' treatment the solution was renewed twice during this period in order to avoid a shift in the pH. In two experiments the samples from pH 5 were accidentally lost during the analysis. Nevertheless the auxin content in the other portions are published, because in both cases two simultaneous treatments at different pH values were undertaken.

Table 1. *The IAA content of wheat root extracts at different pH values of the root culture medium.* The wheat plants were grown at pH 5 for three days. They were then treated at different pH values during varying periods of time. The amount of roots used for extraction was in every treatment 20 g fr. w. The IAA content was measured by means of the Avena cylinder test. Initial Avena section length 5.0 mm.

Root treatment		Avena section length in mm		
hours	at pH	Test control A	Extract added B	Increase in growth B—A
1/2	5	6.04	6.43	0.39
	7.5	6.04	6.41	0.37
1	5	6.04	6.12	0.08
	7.5	6.04	6.17	0.13
1 1/2	5	5.77	6.04	0.27
	7.5	5.77	6.11	0.34
2	6	5.72	6.11	0.39
	7.5	5.72	6.30	0.58
6	6.5	5.44	5.64	0.20
	7.5	5.44	5.86	0.42
12	5	5.53	6.16	0.63
	6	5.53	6.28	0.75
	7.5	5.53	6.32	0.79
24	5	5.72	6.03	0.31
	6	5.72	6.04	0.32
	7.5	5.72	5.97	0.25

After the treatments the whole roots were cut off. Twenty grams of these, fresh weight, were extracted in darkness at 20°C for three hours and a half with 200 ml peroxide-free ether. The root material was not crushed. The methods used for the purification of the ether extracts, the running of chromatograms as well as the measuring of the amount of IAA by the Avena cylinder test are the same as those earlier described by Fransson and Ingestad (1).

All experimental results are collected in Table 1. From this the following can be abstracted:

1. An increase in the pH value of the nutrient solution is followed by an increase in the content of the short-time extractable IAA in the roots.

2. An increase from pH 5 to pH 7.5 involves a more pronounced response than the lower increase from 5 to 6 or from 5 to 6.5.

3. The increase of IAA could be established after a treatment of one hour but seems to be greatest in the 6 hour experiments. If the treatment is maintained for 12 hours, the IAA increase is again diminished and at 24 hours the original level seems to be restored.

The opinion that the indoleacetic acid is an intermediate in the oxidation of tryptophane seems to be well established. The problem has recently been

reviewed by Larsen (6) and Gordon (3). Consequently the amount of auxin present in a plant organ must be dependent not only on the conversion of tryptophane but also on the destruction of the IAA. Both these processes have been shown to be of enzymatic nature. Enzymes which catalyse the tryptophane conversion have been isolated and shown to have a pH optimum of about 7 to 8 (5, 11). This reaction apparently requires oxygen (11). The indoleacetic acid oxidase has been demonstrated in several plant materials. Even this enzyme requires oxygen (8). As to the pH optimum different values have been reported. The enzyme isolated from pea and bean roots was shown to have a optimum between 6 and 7 (8, 10), but, on the other hand, enzymes from pineapple and from Omphalia had an optimum at pH 3.5 and were practically inactive above pH 6 (4, 7). Finally, an indoleacetic acid-destroying enzyme from Polyporus showed a pH optimum of 4.5 (9).

The increase of the IAA content in wheat roots seems to be in good accordance with the tryptophane conversion, and the highest contents are obtained at the pH optimum for this process. Therefore it seems possible that the increase in IAA is a consequence of an increased production. If this is true, it implies that an enzymatic process of the cells in an intact plant organ can be influenced by the external pH value. Against this the objection can be raised that it is hardly possible to change the pH value within the cell. This may be true, but there are many findings, supporting the opinion that the auxin activity is localized to the cell wall or to the surface of the protoplast. In such a case the localization of the auxin synthesis to the same region would not be astonishing and the possibility of influencing the process by means of external conditions understandable. The fact that the increase could be recorded already after one hour also speaks in favour of this opinion.

The destruction of IAA by enzymatic oxidation must be considered as insufficiently known, which is shown from the discrepancies between the investigations of the IAA oxidase. Therefore, even if the increase of IAA after treatment periods of 12 and 24 hours seems to be well established, it is very difficult to interpret. Galston and Dalberg (2) have claimed that the indoleacetic acid oxidase is a highly adaptive enzyme, the presence of which occurs as a response to the amount of auxin present in a plant organ. However, all enzyme preparations which have been investigated are practically inactive at pH values above 7. None of them can call forth a decrease of IAA, similar to that obtained in our analysis. Therefore it must be stated that the decrease after 12 and 24 hours' treatment cannot be explained from present knowledge.

Summary

An increase in the short-time extractable amount of IAA from wheat roots after an increase of the pH value in the nutrient solution could be established. The increase was recorded in roots which were previously cultivated in pH 5 and treated in a solution of pH 7.5 for one hour. If the roots were maintained in solutions with a high pH value for 24 hours, the IAA content was again decreased.

The increase of auxin obtained is in agreement with the enzymatic conversion of tryptophane to IAA. Therefore it is suggested that the increase of the IAA is called forth by an increased production of IAA and that the tryptophane-converting system is localized to some part of the cell, which can be influenced by external conditions.

References

1. Fransson, P. & Ingestad, T.: The effect of an antiauxin on the indoleacetic acid content in Avena coleoptiles. — Physiol. Plant. 8: 336. 1955.
2. Galston, A. W. & Dalberg, L. Y.: The adaptive formation and physiological significance of indoleacetic acid oxidase. — Am. J. Bot. 41: 373. 1954.
3. Gordon, S. A.: Occurrence, formation and inactivation of auxins. — Ann. Rev. Plant Physiol. 5: 341. 1954.
4. Gortner, W. A. & Kent, M.: Indoleacetic acid oxidase and an inhibitor in pineapple tissue. — J. Biol. Chem. 204: 593. 1953.
5. Henderson, J. H. M. & Bonner, J.: Auxin metabolism in normal and crown gall tissue of sunflower. — Am. J. Bot. 39: 444. 1952.
6. Larsen, P.: Formation, occurrence and inactivation of growth substances. — Ann. Rev. Plant Physiol. 2: 169. 1951.
7. Ray, P. M. & Thimann, K. V.: The destruction of indoleacetic acid. I. Action of an enzyme from Omphalia flavida. — Arch. Biochem. 64: 175. 1956.
8. Tang, Y. W. & Bonner, J.: The enzymatic inactivation of indoleacetic acid. — Arch. Biochem. 13: 11. 1947.
9. Tonhazy, N. E. & Pelczar, M. J.: Oxidation of indoleacetic acid by an extracellular enzyme from Polyporus versicolor and a similar oxidation catalyzed by nitrite. — Science 120: 141. 1954.
10. Wagenknecht, A. C. & Burris, R. H.: Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. — Arch. Biochem. 25: 30. 1950.
11. Wildman, S. G., Ferri, M. G. & Bonner, J.: The enzymatic conversion of tryptophane to auxin by spinach leaves. — Arch. Biochem. 13: 131. 1947.

Experiments on the Effects of Neutron-irradiation on the Respiration of Barley Seeds I. Effects on Growth and Oxygen-uptake in Barley Seedlings of Different Varieties

By

Knut Mikaelsen, Ian-Petter Bjørnseth, and Henrik Halvorsen

Institute of Genetics and Plant Breeding, Vollebekk, Norway, and
Botanical Laboratory, University of Oslo, Oslo, Norway
(Received September 17, 1956)

Introduction

Mikaelsen and Halvorsen (1953) have demonstrated that subjection of barley seeds to X-rays decreases the respiration rate of the germinating seedlings. In their experiments the seeds were germinated in petri-dishes, and their growth and rate of respiration were measured daily during the first week of germination. It was found that doses of 5,000––15,000 r. did not affect the initial increase in rate of respiration but that after 4 or 5 days the respiration of irradiated seedlings proceeded markedly more slowly than in the untreated control seedlings. Measurements of seedling length indicated that the radiation had caused a corresponding retardation of growth. A dose of 2,500 r. had no appreciable influence on respiration or on growth.

This preliminary study stimulated further investigations concerning the effects of ionizing radiations on respiration and respiratory mechanisms.

Material and Methods

Dormant seeds of the barley varieties Jotun, Varde, and Domen, were used as experimental material. Jotun and Varde are six-rowed, whereas Domen is a two-rowed variety. The water-content of all the seeds was approximately 10 per cent at the start of the experiments. The dry, dormant seeds were

subjected to irradiation in the centre of the isotope channel $1/7$ in the heavy-water reactor, Jeep, run by the Joint Establishment of Nuclear Energy Research at Kjeller, Norway. The seeds were placed in cylindrical aluminium tubes, and enclosed in cylindrical graphite boxes, whose walls were 5 mm. thick.

Since the radiation in the centre of the pile comes from various sources, a clear description and definition of these sources is necessary. There are present at the centre thermal neutrons of different energies, non-thermal neutrons, and gamma-radiations. The doses of the various kinds of irradiation have been determined by chemical dosimetrical methods (Sæland and Ehrenberg, 1952; Ehrenberg and Sæland, 1954 a and b). The main part of the radiation at the centre consists of neutrons which have reached thermal equilibrium with the moderating atoms. They have a Maxwellian velocity distribution around 0.025 eV. The dose contribution from the thermal neutrons is due mainly to the secondary particles formed in nuclear reactions. These particle radiations produce the following ion densities: the 0.56 MeV protons formed in N^{14} (np) C^{14} give about 2300 ion pairs/μ and the alpha particles from B^{10} (nγ) Li^7 give about 6000 ion pairs/μ. When lithium is present in the tissue, a less densely ionising triton is formed, giving about 1300 ion pairs/μ in addition to the alpha particle.

The reactor was operated at a low level of 10 KW, which was most convenient for the doses applied. At this level the flux of thermal neutrons was 1.2×10^{10} n_{th}/cm²/sec. When biological material is irradiated in the centre of the reactor, the dose contribution from non-thermal neutrons is usually more important than that from thermal neutrons. The non-thermal-neutrons transfer energy to the tissue mainly by elastic collisions with the atoms of the tissue. In order to estimate the magnitude of this contribution it is necessary to have a general understanding of the flux of non-thermal neutrons as a function of neutron energy. For energies up to 0.1 MeV, the non-thermal flux may be regarded as inversely proportional to the energy. This "1/E law" may be extended to 1 MeV, although here it is only approximate. Beyond 1 MeV the the neutron spectrum, in the irradiation position used, proved essentially to be that of unmoderated fission neutrons, except that the relative number of very high energy neutrons was reduced (Ehrenberg and Sæland, 1954).

Compared with the rather discrete types of radiation originating from the capture of thermal neutrons, non-thermal neutrons produce a wide range of ion densities due to protons scattered by neutrons of energies from thermal to about 10 MeV. Thus it is impossible to give a definite ion density for the non-thermal neutrons. The ion densities for these energies are estimated at 1300—3000 ion pairs/μ, and these are regarded as typical of tissue doses

from non-thermal neutrons in the pile. The flux of fast neutrons is approximately (1.4 ± 0.2) 10^9 n/cm²/sec. at the pile position used.

The contaminating gamma radiations in this position in the pile originate from different sources, such as the spontaneous gamma radiation of the fission process, radioactive fission products, and (n, γ) reactions and induced radio-activity in the pile materials. Some gamma radiations originate from the irradiated object itself, chiefly from the H (n, γ) D reaction occurring within the material. The effects of the "internal" gamma radiation are almost negligible in relation to the effects of the neutrons. Even the external gamma radiation contributes only insignificantly to the total radiation, a maximum of 10 per cent. With its sparse ion density of 8 ion pairs/μ the effects of gamma radiation may almost be disregarded.

The samples of seeds were irradiated in the centre of the pile, and exposed to doses of thermal neutrons ranging from $(0.5 \times 10^{12}$ to $6 \times 10^{12})$ n_{th}/cm². The doses are expressed as the number of thermal neutrons per cm², although the main biological effects are caused by the accompanying epithermal and fast neutrons. According to Ehrenberg and Sæland's calculations (1954 b) the non-thermal neutrons are responsible for 70—80 per cent of the total dose when barley seeds are irradiated in the centre of the pile. On the basis of these calculations, the doses can be estimated in reps. If the calculations are applied to the barley material used in these experiments, then the seeds have been exposed to neutron-irradiations of doses ranging from 300—3,600 reps.

Experiment No. 1. The first radiation treatments were carried out in June 1954. Seeds, from 1953, of the Jotun variety were used as experimental material. Immediately after their exposure to radiation the seeds were placed, with no previous soaking, on moist filter paper in petri-dishes, and left to germinate in darkness at a temperature of 20°C. In order to prevent fungal infection, the seeds were first washed in desinfectant. Infected seeds were not used in the experiments.

In December, 1954, samples of seeds from 1954 of the Jotun, Varde, and Domen varieties were irradiated in the same position in the pile and with similar doses of neutrons.

Experiment No. 2. In December 1954 the same experiment was carried out with seeds of the Domen variety. The seeds were treated with a mercury dust (Ceresan) before the radiation exposures.

Experiment No. 3. The Varde variety was used in this experiment. The seeds were soaked in dilute hypochlorite before being laid out for germination and respiration measurements in January 1955.

Experiments Nos. 4 and 5. In these experiments seeds of the Jotun and the Domen varieties were studied simultaneously. The seeds were treated for 16 hours with a mercury dust ("Abavit") before germination. The experiments were performed in February.

All the seeds were soaked in water for three hours before the experiments were

begun. The seeds were then laid out to germinate on moist filter paper in aluminium trays (size 28×42 cm.), which were covered with glass plates.

The rate of respiration of both untreated and irradiated seeds was measured daily during the first week of germination. A modified Warburg constant-volume respirometer was used — as in the X-ray experiment carried out by Mikaelsen and Halvorsen (1953). The apparatus and technique are described in greater detail elsewhere (Halvorsen, 1955). Six or seven days after germination the length of the seedlings exceeded that of the respirometer flasks, and the seedlings had to be bent gently when placed in the flasks. After the seventh day, masurements could be made only with great difficulty owing to unavoidable damage to the seedlings.

The respiration rate (gas exchanged) was measured at $20°C$ and was expressed in terms of μl $O_2/100$ mg. dry weight and hour. A number of seedlings, ranging from 5—10 according to the stage reached in germination, was placed in the respirometer flask for each measurement. The manometer experiments were continued for four hours, and the mean respiration rate per hour was determined. Four parallel analyses of each series of experiments were carried out except in the December 1954 experiment with the Domen variety, where five parallel samples were analysed.

The effects of radiation on growth were determined on the basis of measurements of seedling length. 50 seedlings of each series were measured on the 3rd, 5th, and 7 (8)th day after germination.

Experimental Results

The seed samples of the different varieties were selected carefully. Under the germination control at the State Seed Testing Station, Vollebekk, Norway, the seeds showed 90—100 per cent germination. In experiment no. 1 the seeds were almost one year old when used in the respiration experiments. After one year it was found that the germinative capacity of the seeds was reduced to 70 per cent for some unknown reason.

The germination of the 50 seeds in each treatment was studied. Doses of neutrons ranging from (0.5×10^{12}) to (6×10^{12}) n_{th}/cm^2 had no appreciable effect on the initial germination; except in Exp. no. 1 where the irradiated seeds germinated better than the untreated controls (70 per cent) (Table 1).

The seedling growth, however, was strongly influenced by the neutron radiation. Although the germinative capacity of the seeds was not effected,

Table 1. *Per cent germination in untreated and neutron-treated barley seeds after 3 and 5 days of germination.*

Radiation dose n_{th}/cm^2 in reps		0	0.5×10^{12} 300	10^{12} 600	2×10^{12} 1,200	3×10^{12} 1,800	4×10^{12} 2,400
% germination after	3 days	70	85	78	63	70	80
	5 days	70	81	78	69	70	80

Table 2. *Relative seedling length of neutron-irradiated barley seeds.* (Control=100). Radiation doses given in n_{th}/cm^2 (upper row) and reps. (lower row).

Experiments and time	0.5×10^{12} 300	10^{12} 600	2×10^{12} 1,200	3×10^{12} 1,800	4×10^{12} 2,400	6×10^{12} 3,600
Experiment No. 1.						
After 3 days	44	40	28	32	24	--
„ 5 „	77	74	62	51	34	—
„ 7 „	94	90	77	50	30	—
„ 8 „	108	101	85	51	29	—
Experiment No. 2.						
After 3 days	—	77	85	69	43	29
„ 5 „	—	81	87	63	49	25
„ 7 „	—	92	88	66	47	21
„ 9 „	—	93	78	48	37	14
Experiment No. 3.						
After 3 days	—	50	—	59	—	26
„ 5 „	—	94	—	62	—	26
„ 7 „	—	104	—	70	—	20
Experiment No. 4.						
After 3 days	—	39	—	89	—	7
„ 5 „	—	82	—	72	—	15
„ 7 „	—	85	—	64	—	12
Experiment No. 5.						
After 3 days	—	97 →	—	66	—	5
„ 5 „	—	83	—	50	—	13
„ 7 „	—	103	—	63	—	9

a pronounced inhibition of growth in the irradiated seeds was noticed on the third day, and the reduced growth appeared unchanged throughout the experimental period (Table 2). The retardation of seedling growth increased with increasing dose. Figure 1 illustrates the relative seedling lengths on the 7th day after germination, showing that the reduction is almost linearly proportional to the dose. The curves (Figure 1) indicate that the differences between the materials from the different experiments are negligible, and so no differences between the separate varieties, regarding the effects of neutron irradiation on growth can be established.

The results of the measurements of rate of respiration of untreated and irradiated seeds are plotted in graph form in Figures 2—6. The respiration of the untreated control seeds proceeded at an increasing rate throughout the experimental period in all experiments.

No differences were found between untreated and irradiated seedlings on the first and second day after germination. From the third day pronounced differences were to be seen between the two.

From the diagrams in Figures 2—6 it appears that doses of (0.5×10^{12}) and 10^{12} n_{th}/cm^2 had very little or no effect on the respiration rate throughout
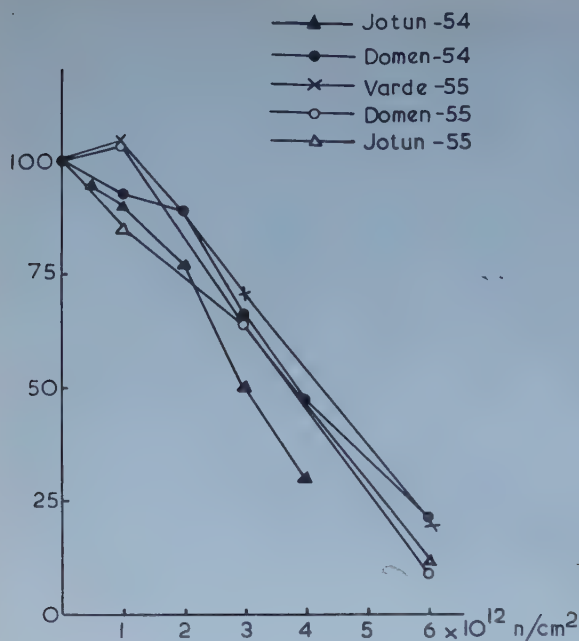
Figure 1. *Relative lengths, measured on the 7th day after germination, of the seedlings irradiated with various doses of neutrons.* (Control=100).
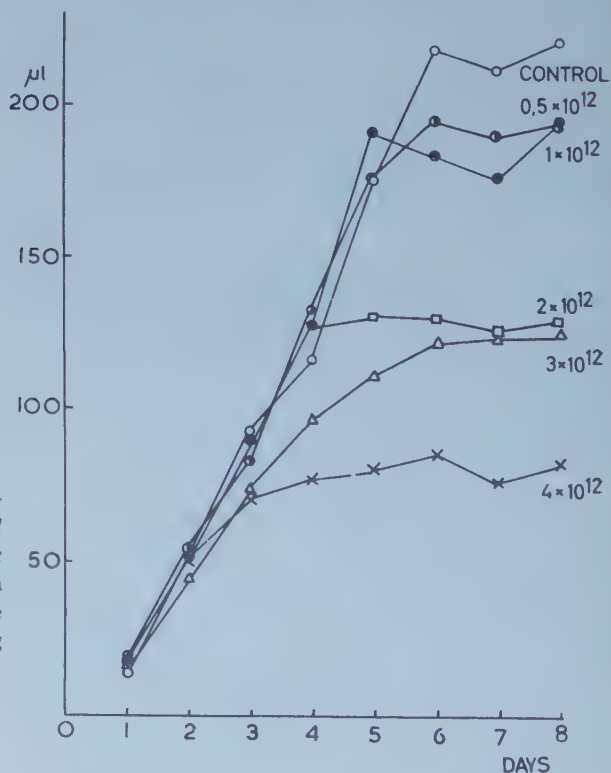

Figure 2. *Respiration of untreated and neutron-irradiated seedlings during the first week after germination.* On the abscissa time in days, on the ordinate respiration rate in μl O_2/100 mg and hr.
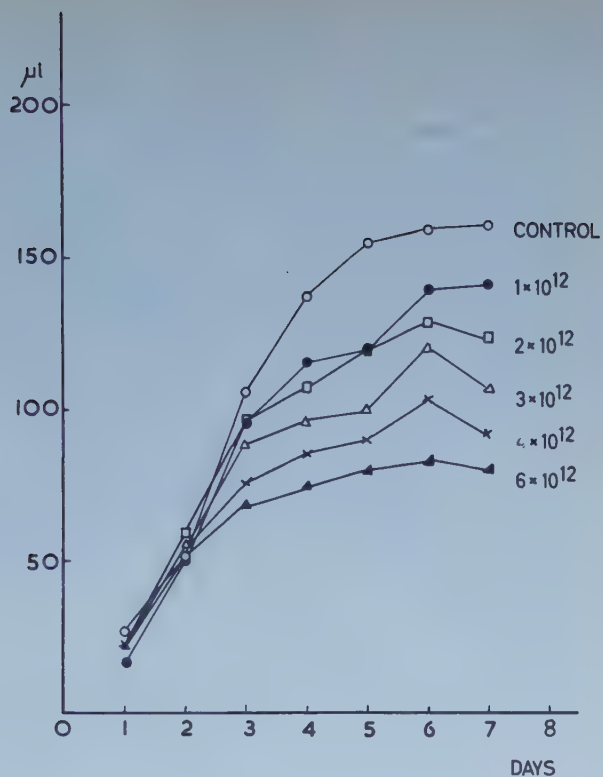
Expt. no. 1 Jotun -54.
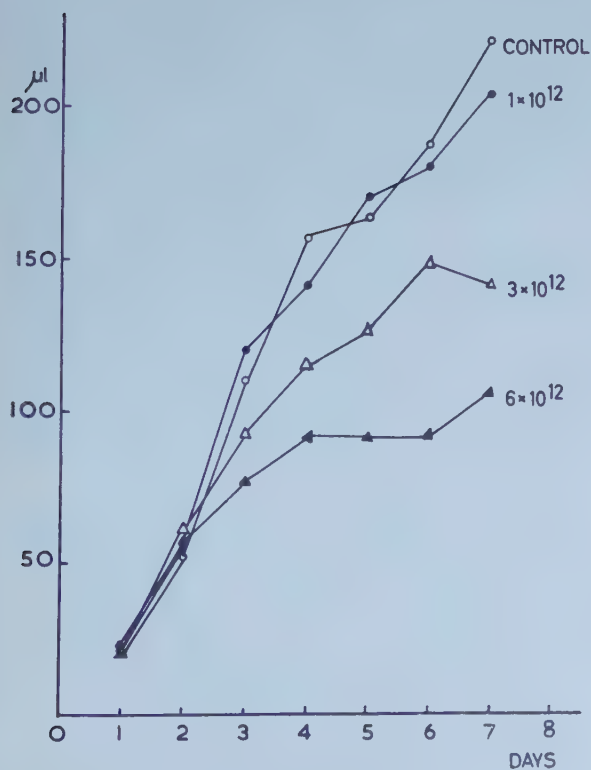
Figure 3. See text on Figure 2.
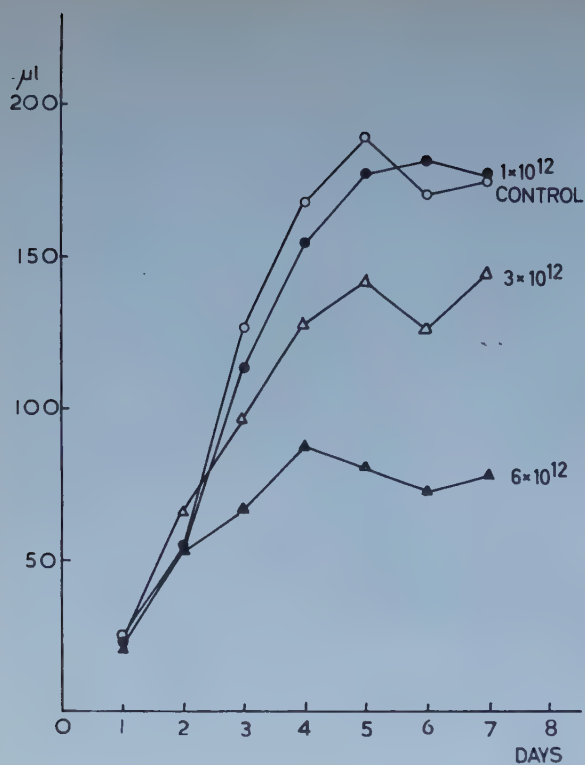Expt. no. 2. Domen -54.

Figure 4. See text on Figure 2.
Expt. no. 3 Varde -54.

Figure 5. See text on Figure 2.
Expt. no. 4 Domen -55.
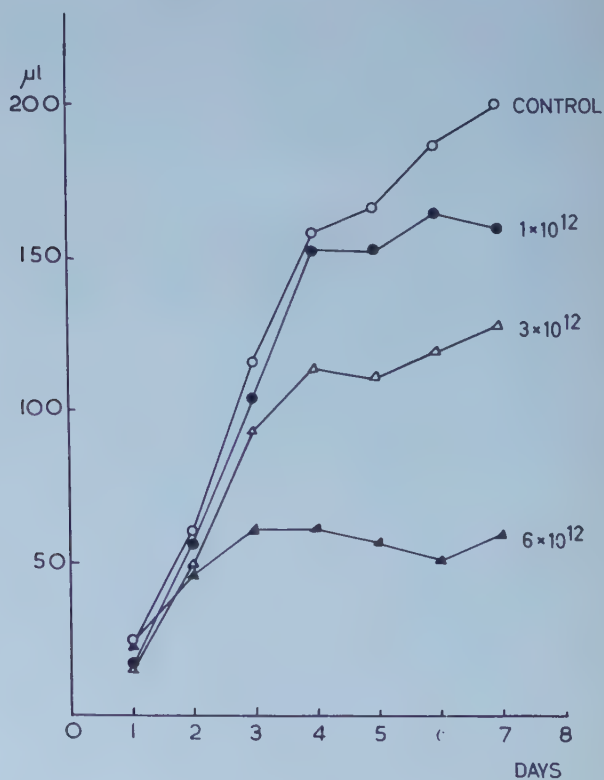
Figure 6. See text on Figure 2.
Expt. no. 5. Jotun -55.
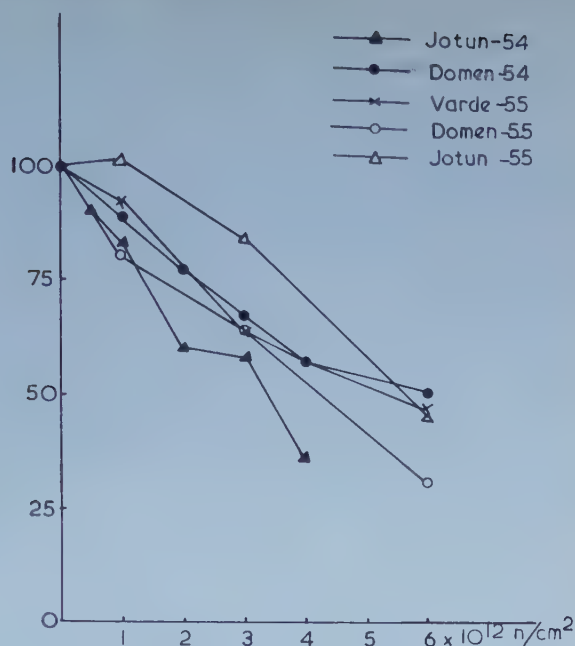
Figure 7. *Relative respiration of irradiated seedlings* (control = 100) *on the 7th day after germination. The curves indicate the relationship between dose and respiration decrease*

the experimental period Large doses had a marked retarding effect on respiration. The effects of radiation increased from the 3rd to the 7th day.

The diagrams in Figure 7 demonstrate the relative respiration r tes of irradiated seeds on the 7th day.

It seems that in these experiments there was far more variation n the effects on respiration than on growth (cf. Figures 1 and 7). A reg ession analysis revealed the following: —

1. The variations in the regression coefficients ($\hat{\beta}$) in the different experiments seem far too great to allow the computation of a basic common regression line around which the values would be distributed at random (see Table 3).

2. In some cases there were significant deviations from linearity in the regression curves. Therefore, a general linear relationship cannot be assumed to exist between the neutron-dose and the rate of respiration.

Table 3. *Regression coefficients showing the effect of increasing radiation doses on the relative respiration rates of three barley varieties.*

Expt.	Jontun 54	Jontun 55	Domen 54	Domen 55	Varde 55
$\hat{\beta}$	— 32.6	— 16.8	— 13.7	— 22.1	— 20.1

Since the respiration values are relative figures (respiration per unit of dry weight), corrections for the varying dry weights were introduced in the statistical computations.

It would be of interest to know whether there are any real differences in the sensitivity to radiation of the separate varieties. Since different experiments with the same variety seem to give widely different results (*e.g.* Jotun and Domen in Table 3), a comparison including all the experiments would be of small value.

Experiments nos. 4 and 5 with Domen and Jotun were carried out at the same time, in February 1955, and all controllable conditions should have been the same in both cases. Since the respiration rate was different for the two varieties, the relative decreases must be compared in order to determine the different responses of the two varieties to radiation. The effects may be defined as equal when

$$\frac{J_j}{J_o} = \frac{D_j}{D_o} \quad (j = \text{radiation dose})$$

A statistical test of these proportions was made by logarithmic transformation. It was found that the variety Varde could also be included in the comparison since the experimental conditions were almost identical for the three experiments.

The regression coefficients which were calculated for each of the three varieties on the assumption of a linear regression, were different (Table 4). Significant deviations from linear regression were found to occur in two of the three varieties. Consequently no common basic linear regression can be supposed to exist. However, a common regression line can be computed for the sake of comparison. Analysis of the distribution of the points representing each of the three varieties around this line gives significant differences $(P < 0.001)$. This indicates that the relative reduction in the respiration rate of these varieties is different (Figure 7).

Thus, it may seem that the two-rowed variety, Domen, was more seriously affected than the six-rowed varieties, Jotun and Varde. In the X-ray experiments (Mikaelsen and Halvorsen, 1953) the two-rowed variety, Maja, was

Table 4. *Analysis of variance of three of the experiments included in Table 3.*

Variety	S_β	$\hat{\beta}$	V_β	F
Jotun —55	967.5	— 60.68	13.105	13.5 ***
Varde —55	1,399.9	— 56.48	2.836	2.03
Domen —55	1,346.0	— 85.25	8.107	6.02 *

Figure 8. *Percentage of water in untreated and irradiated seedlings during the first week after germination.* On the ordinate water content in per cent of fresh weight.
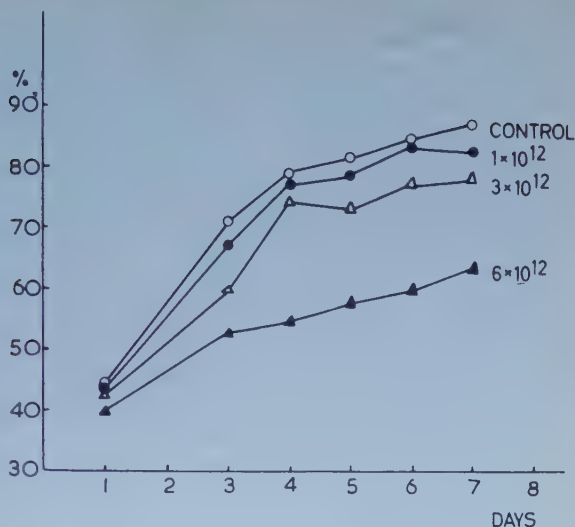
less radio-sensitive than the six-rowed variety, Jotun. No significant differences could be established between Jotun and Varde.

The water-content of the seeds was determined daily during the experimental period. Results from one of the experiments (no. 5, Domen-55, are plotted in Figure 8). The water-content of the irradiated seeds was reduced as compared with the untreated controls. It is seen that the effect was particularly pronounced at a dose of 6×10^{12} n_{th}/cm^2.

Discussion

The results of the experiments demonstrated clearly that respiration was markedly impaired by neutron radiations, as had been the case when barley seeds were exposed to X-rays (Mikaelsen and Halvorsen 1953). It has been found that X-rays have a similar effect on wheat seedlings (Francis, 1934).

After both neutron and X-irradiation it seemed that respiration was unaffected for the first 2—4 days after germination. Since this typically delayed effect was observed in both cases, it may be concluded that the respiratory systems and mechanisms were unimpaired by ionizing radiations, while it was the synthesis of important products which had been seriously affected by them. Therefore, during seedling growth there may be a deficiency of some of these products because their synthesis has been blocked. The result is a reduction in the rate of respiration. In the following paper (part 2) this matter will be discussed in greater detail.

46

The effects of ionizing radiations on respiration is obviously complex, as demonstrated by the deviating results obtained. Until recently it has been assumed that ionizing radiation interferes only to a minor extent with respiration. Sussman (1953), however, found that in potato tubers Q_{O_2} and Q_{CO_2} increased following X- and gamma-radiation over a wide range of dosages, but no accompanying changes in cytochrome oxidases or tyrosinase were observed. In Escherichia coli, Billen *et al.* (1953) demonstrated that in suspensions exposed to an X-ray dose of 60,000 r. no effects on the initial respiratory rate was apparent. But this initial period was followed by a period of marked decline in oxygen consumption. After this high dose of X-rays, 99 per cent of the cells were killed. The complex effects of radiation are further demonstrated in Arbacia eggs (Barron and Seki, 1953). After small doses an increase in rate of respiration was evident.

Thus it seems that it is important now to investigate the metabolic processes which underly the visible effects on the rate of respiration.

It was demonstrated that the relationship between respiration and neutron dose deviated very little from linearity (Figure 7). In the X-ray experiments (Mikaelsen and Halvorsen, 1953) the dose curves had a pronounced and typically sigmoid shape. Such a different effect of the two types of radiation may be explained by their different physical properties. The neutrons exert their effects through very dense ionizing particles in contrast to the more sparsely ionizing X-rays.

Thus the greater part of the deviations in the dose curves may be due to the different ion densities. Some authors (Caldecott *et al.*, 1952; MacKey, 1952, 1954; Mikaelsen and Aastveit, to be published) have emphasized the possibility that the two types of ionizing radiation — neutrons on the one hand, and X-rays or γ-rays on the other — may exert their biological effects through different mechanisms. A discussion of this problem may be postponed, however, since additional material from studies concerning the effects of neutrons and X-rays will be published later.

Here only a few characteristic features of the relationship between growth and respiration in the neutron and X-ray experiments need be mentioned.

After X-irradiation (Mikaelsen and Halvorsen, 1953) it was impossible to establish any relation between the reduction in growth and the decrease in respiration rate. Cytological examinations seemed to indicate that there was no direct correlation between the reduction in growth or respiration and the number of cells with damaged or injured nuclei. In the neutron-irradiated material, however, the reduction in growth was proportional to the decrease in respiration, and also to the frequency of damaged cell nuclei (as seen under cytological examination). Details of the cytological and anatomical analyses are given in a following paper (part 2).
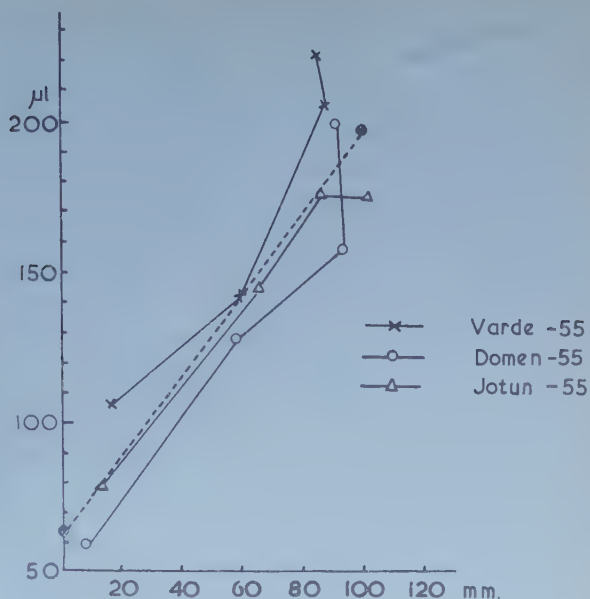
Figure 9. *The relationship between respiration and seedling length in the varieties Varde Domen and Jotun.* The joint regression line $Y = 62.5 + 1.4 \cdot X$ is plotted in the figure (- - - - -). On the abscissa seedling length in mm., on the ordinate respiration in μl $O_2/100$ mg and hr.

Altogether these facts may be taken to indicate that the biological effects caused by neutron irradiation are due mainly to their effect on the cell nuclei, while those caused by X-rays are to a greater extent due to extra-nuclear or cytoplasmic disturbances.

Since the reduction in growth was proportional to the decrease in respiration it would be of interest to examine the relationship between respiration rate and seedling length. In Figure 9, where the rate of respiration is plotted as a function of seedling length, the positive correlation between the two is clearly illustrated.

Since the estimated regression coefficients for the three varieties were not significantly different, the same positive correlation between seedling length and rate of respiration is shown in all the experiments. The fact that the respiration rate can be correlated with seedling length indicates that the respiration deficit may be a consequence of the reduced or inhibited seedling growth. This necessitates a reconsideration of the experimental methods. This problem will be discussed in Part 2 of this paper.

There are no clear explanations of the reduced water content of irradiated seedlings which was seen in these experiments. The reduced plant size and seriously injured roots of the neutron-irradiated seedlings could account for it. It is also conceivable, however, that the reduced water content was due to the decreased metabolic activities in irradiated seedlings. In experiments with barley and rye (unpublished) it has been found that neutron irradiated

seedlings have a larger dry matter content than the normal seedlings. This was independent of seedling length except in very small plants. Similar results were obtained by Ehrenberg and Wettstein (1955). A further discussion of this problem is given in the next paper (part 2).

Statistical analyses revealed significant differences between the varieties Varde, Jotun, and Domen concerning the effect of neutron radiation on decrease in rate of respiration. In the X-ray experiments (Mikaelsen and Halvorsen, 1953) the six-rowed variety Jotun proved more radio-sensitive than the two-rowed variety Maja. Unfortunately there were no seeds of the latter variety of good enough quality to be used in the neutron experiments.

Summary

Barley seeds of the varieties Jotun, Varde, and Domen were irradiated in the centre of an atomic reactor and exposed to different doses of neutrons.

The germinative capacity of the seeds was not affected by the radiation, but seedling growth was seriously impaired and the retardation increased with dose.

The respiration rate, measured as oxygen uptake par unit dry weight, was unimpaired by radiation during the first two days after germination. From the third to the seventh day there appeared a pronounced reduction in rate of respiration. This reduction increased with increasing radiation dose.

A positive correlation between respiration and seedling length was demonstrated.

Throughout the experimental period the water content of the irradiated seedlings was lower than in the untreated control seedlings.

References

1. Barron, E. S. G. & Seki, S. L.: Studies on the mechanism of action of ionizing radiations. VII. Cellular respiration, cell division and ionizing radiations. — J. Gen. Physiol. 35: 865. 1952.
2. Billen, D., Stapleton, G. E. & Hollaender, A.: The effect of X-radiation on the respiration of Escherichia coli. — J. Bacteriol. 65: 131. 1953.

3. Caldecott, R. S., Frolik, E. F. & Morris, R.: A comparison of the effects of X-rays and thermal neutrons on dormant seeds of barley. — Proc. Nat. Acad. Sci. (U.S.) 38: 804. 1952.

4. Ehrenberg, L. & Sæland, E.: Chemical dosimetry of radiations giving different ion densities. An experimental determination of G values for Fe^{2+} oxidation. — Jener publ. No. 8, 1954.

5. — — Effects of pile radiation on barley seeds. — J. Nucl. Energy, 1: 150. 1954.

6. — & von Wettstein, D.: Radiation effects on the dry matter content in barley. — Bot. Not. 108: 216. 1955.

7. Francis, D. S.: The effects of X-rays on growth and respiration of wheat seedlings. — Bull. Torr. Bot. Club. 61: 119. 1934.

8. Halvorsen, H.: The gas exchange of flax seeds in relation to temperature I. Experiments with immature seeds and capsules. — Physiol. Plant. 8: 501. 1955.

9. MacKey, J.: The biological action of X-rays and fast neutrons on barley and wheat. — Ark. Bot. Ser. 2, 1: 545. 1952.

10. — Neutron and X-ray experiments in wheat and revision of the speltoid problem. — Hereditas 40: 65. 1954.

11. Mikaelsen, K. & Halvorsen, H.: Experiments on the respiration of X-irradiated barley seeds. — Physiol. Plant. 6: 873. 1953.

12. — & Aastveit, K.: A comparison between the effects of neutrons and chronic gamma radiation on growth and fertility in oats and barley. — (in manuscript).

13. Sussman, A.: The effect of ionizing radiation upon the respiration and oxidases of the potato tuber. — J. Cell. Comp. Physiol. 42: 273. 1953.

14. Sæland, E. & Ehrenberg, L.: A differential dosimetry of pile radiations using Fe^{2+} oxidation. — Acta Chem. Scand. 6: 1133. 1952.

Growth Inhibiting Substances Formed by Algae

By

Erik G. Jørgensen

Botanical Department, Royal Danish School of Pharmacy, Copenhagen
(Received November 10, 1956)

1. Introduction

During investigations in two Danish lakes the author has studied the relationship between the growth of epiphytic and planktonic diatoms (Jørgensen 1957). It was shown that during the maxima of planktonic diatoms only few epiphytic diatoms were found and that the maxima of epiphytic diatoms occurred only when the planktonic diatoms were present in small quantities. It was shown further that in the lake Lyngby Sø, where a great production of phytoplankton, especially green algae, took place during most of the year, the production of epiphytic diatoms was much smaller than in the lake Furesø, where the production of phytoplankton was considerably smaller. Differences in light conditions for the epiphytic diatoms were without importance. It was concluded that the growth of the epiphytic diatoms in the above cases was inhibited by substances produced by the planktonic diatoms and green algae.

The presence of growth-inhibitors in algae cultures and in natural waters has previously been discussed by different authors. In this paper experiments with different algae are presented to elucidate the problem.

2. Materials and methods

In the experiments the following algae were used:

Nitzschia palea (Kütz.) W. Smith, an epiphytic freshwater diatom isolated from the eutrophic lake Lyngby Sø near Copenhagen in Dec. 1949 and used by the author in previous experiments (Jørgensen 1953 and 1955).

Asterionella formosa Hass. I, a planktonic freshwater diatom isolated from the eutrophic lake Furesø near Copenhagen in April 1954.

Asterionella formosa Hass. II, isolated from Lyngby Sø in January 1956.

Scenedesmus quadricauda Breb., planktonic green algae isolated from Lyngby Sø in January 1956.

Chlorella pyrenoidosa Chick, planktonic green algae. The strain was obtained through the kindness of Professor Harder at the Botanical Institute in Göttingen and was isolated in 1944 in the botanical garden there. It was cultivated by the author since 1952.

In all experiments a modification of Rodhe's culture solution VIII (Rodhe 1948) was used. The following salts in mg/l were used: $Ca(NO_3)_2$, 60.0; $MgSO_4$, 5.0; $MnSO_4$, 0.03; Na_2HPO_4, 5.0; $KHCO_3$, 200.0; ferric citrate-citric acid, $1.0+1.0$; soil extract, 10 ml/l and silicate as sodium or potassium silicate, 5.0 mg Si/l. The distilled water was purified by passage through a column of Amberlite I R-100 (hydrogen form). The water containing silicate, $Ca(NO_3)_2$, $MgSO_4$ and $MnSO_4$ was heated to 100°C and after cooling Na_2HPO_4, ferric citrate-citric acid and soil extract were added from separate heated stock solutions. $KHCO_3$ was added as crystals. The pH of the culture solution was 8.5.

After suitable dilutions the cells of the organisms — except Chlorella — were sedimented in special chambers (volume 2.6 ml) with cover glasses as bottoms and were counted by means of an inverted microscope as described by Utermöhl 1931. There was no problem with the counting of Asterionella and Scenedesmus. The diatom Nitzschia palea presents a problem in that it sticks firmly to the bottom of plastic, though not pyrex, vessels. It is necessary to detach them from the bottom with conc. H_2SO_4. In pyrex flasks some of the diatoms similarly stick to the bottom although others do not. When Nitzschia from pyrex flasks was counted it was necessary first after shaking to count the free cells in the culture solution next to treat the fixed diatoms at the bottom with conc. H_2SO_4 and then count them. It is sufficient to treat the bottom with H_2SO_4 for 24 hours. A treatment of longer duration destroys the chromatophores and separates the two frustules of the cell, which makes it more difficult to count the cells.

Counting of the cells of Chlorella was done by using a haemacytometer (Thoma). In each culture the value is the average of five countings of 400 squares (400 sq.= 0.05 mm³).

3. Experiments with two diatoms

For the *first experiment series* \u planktonic and an epiphytic diatom, *Asterionella formosa* and *Nitzschia palea*, were used. The experiments were carried out in a north window without artificial light in April 1955. 12 Erlenmeyer flasks (Sial, 200 ml) containing 50 ml culture solution were inoculated with Asterionella (94,000 cells/flask) and 12 flasks with Nitzschia (107,000 cells/flask). The flasks were shaken every day. After 8 days of culturing new experiments were started with 3 flasks inoculated with Asterionella (120,000 cells/flask) in each lot.

A 1: A control experiment with Asterionella inoculated in a fresh medium.

A 2: An experiment in a fresh medium inoculated with both Asterionella and Nitzschia.

A 3: Asterionella was inoculated in filtrates from three Asterionella cultures in the preliminary experiment. The culture solution was filtered through a glass filter (3 G 3).

A 4: Asterionella was inoculated in filtrates from three Nitzschia cultures.

A 5: The same experiment as A 4 but the filtrates were heated to 100°C. After cooling $KHCO_3$ was added in addition to the nutrient salts mentioned below.

A 6: = N 6.

A 7: Asterionella was inoculated in three living Nitzschia cultures.

To the filtrate respective the culture solutions from the preliminary experiment nutrient salts of silicate, $Ca(NO_3)_2$, Na_2HPO_4 and ferric citrate-citric acid were added in usual quantities in all cases. Corresponding cultures were started with Nitzschia (505,000 cells/flask):

N 1: A control with Nitzschia inoculated in a fresh medium.

N 2: The same as A 2.

N 3: Nitzschia inoculated in filtrates from three Nitzschia cultures.

N 4: Nitzschia inoculated in filtrates from three Asterionella cultures.

N 5: The same as N 4 but the filtrates were heated to 100°C. After cooling $KHCO_3$ was added in addition to the nutrient salts mentioned above.

N 6: Nitzschia was inoculated in three living Asterionella cultures.

N 7: = A 7.

The experiments were discontinued after 7 days of culturing.

Asterionella. The number of divisions per day of Asterionella in the seven experiments is presented in Figure 1. In A 2 where Asterionella and Nitzschia were inoculated together there was no significant difference from the control. When Asterionella grew in a filtrate from an Asterionella culture (A 3) the number of divisions/day were considerably greater than in the control. The cells had divided once more during the 7 days. So Asterionella does not seem to form autotoxic substances. The faster growth rate relative to the control is probably due to a greater content of nutrient salts in A 3 than in A 1 since the filtrate was not exhausted for nutrient salts and new quantities were added. Another possibility is that the filtrate from the first cultures contains substances which accelerate the growth of Asterionella.

Experiment A 4 shows that Asterionella did not grow as well in the filtrate from a Nitzschia culture as in the control. The number of divisions per day were, on the average, 0.52, as compared to 0.65 in the control. When Asterionella was inoculated in a filtrate from a Nitzschia and the filtrate was heated
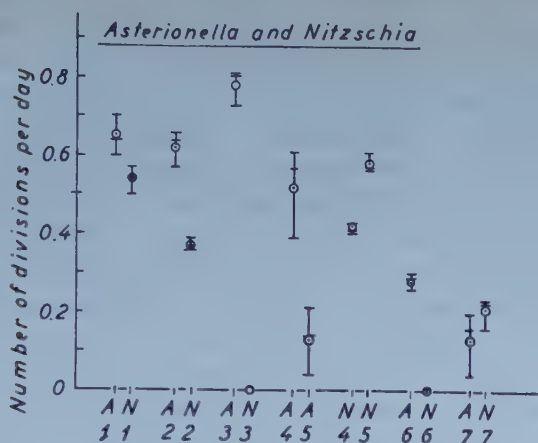
Figure 1. *The number of divisions per day in experiment series 1.*

A 1 and N 1. Controls with Asterionella and Nitzschia, respectively.

A 2 and N 2. Asterionella and Nitzschia growing together, inoculated at the same time.

A 3. Asterionella growing in filtrate from an 8 days old Asterionella culture.

A 4. Asterionella growing in filtrate from an 8 days old Nitzschia culture.

A 5. Same as A 4 but the filtrate was boiled.

N 3. Nitzschia growing in filtrate from an 8 days old Nitzschia culture.

N 4. Nitzschia growing in filtrate from an 8 days old Asterionella culture.

N 5. Same as N 4 but the filtrate was boiled .

A 6 and N 6. Nitzschia inoculated in an 8 days old Asterionella culture with living cells.

A 7 and N 7. Asterionella inoculated in an 8 days old Nitzschia culture with living cells.

The horizontal lines show the values from the individual cultures, the circles the averages values.

to 100°C before inoculation (A 5) Asterionella did not grow as well as in A 4 with an unheated filtrate. The reasons for this remarkable result are not clear.

In A 6 where Nitzschia was inoculated in an 8 days old Asterionella culture, the Asterionella cells continued the growth which, however, was limited by deficiency of nutrient salts owing to the great number of cells and so many new cells were formed as could be expected from the silicate content of the medium.

The growth of Asterionella when inoculated in an 8 days old Nitzschia culture (A 7) was inconsiderable but this was most likely due to the great competition for the nutrient salts with Nitzschia, which was found in much greater numbers.

The conclusions of the Asterionella experiments are that this species does not seem to form autotoxic substances (A 3) but that Nitzschia forms substances which inhibit the growth of Asterionella (A 4).

·*Nitzschia*. The results of the experiments with Nitzschia are also presented in Figure 1. Experiment N 2 where Nitzschia was inoculated together with Asterionella shows a distinct inhibition of the growth of Nitzschia as compared to the control (N 1). The number of divisions per day were 0.54 in the control and 0.37 in N 2. In N 3 where Nitzschia was cultivated in filtrate from an 8 days old Nitzschia culture no growth could be observed. In N 4 where Nitzschia was cultured in filtrate from an 8 days old Asterionella culture an inhibition of the same extent as in N 2 took place. When Nitzschia was cultured in filtrate which was heated to 100°C (N 5) the growth was as good or perhaps a little better than in the control (greater quantities of nutrient salts, compare A 3). This shows that the inhibiting substances formed by Asterionella are destroyed by heating.

When Nitzschia was inoculated in an 8 days old culture of Asterionella (N 6) no growth at all was found. In N 7 Asterionella was inoculated in an 8 days old Nitzschia culture. Both Asterionella and Nitzschia divided about once during 7 days.

It is concluded from the experiments that Nitzschia forms autotoxic substances, and that Asterionella forms substances which inhibit the growth of Nitzschia.

In the first series of experiments some of the results were not unequivocal. Thus in experiment A 2, N 2, where Asterionella and Nitzschia were cultivated together, it was questionable whether the inhibition of the growth of Nitzschia was due only to inhibiting substances, or to competition for nutrient salts as well. In A 7 and N 6 the number of divisions was small. This was caused primarily by a lack of nutrient salts, but some inhibition may also have taken place. Therefore a new series of experiments was started in which nutrient salts were added to the cultures from time to time so as to eliminate any deficiency effect on the growth.

Experiment series 2. The stock culture of Asterionella used in series 1 had died during the very warm summer of 1955. A new strain was isolated from the lake Lyngby Sø.

In first part of the series 28 Erlenmeyer flasks were used each containing 50 ml Rodhe VIII. The flasks were placed in a thermostat bath at 18°C and illuminated constant from below with six tubular fluorescent lamps giving about 6000 lux. 11 flasks were inoculated with Asterionella, (11,700 cells/flask), 11 with Nitzschia (125,000 cells/flask) and 6 with both Asterionella and Nitzschia (same concentration as above). In 3 flasks from each inoculation nutrient salts with Si, Fe, P and N were added every day corresponding to a sixth of the usual content. The first part of the series lasted for 8 days and was composed of the following experiments:

A 1a: Asterionella control.

A 1b: As A 1a but nutrient salts added during the experiment.

A 2a: Asterionella and Nitzschia inoculated together.

A 2b: As A 2a but nutrient salts added during the experiment.

N 1a: Nitzschia control.

N 1b: As N 1a but nutrient salts added during the experiment.

N 2a=A 2a.

N 2b=A 2b.

After 8 days culturing the second part of the series was started.

A 3a=N 3a.

A 3b=N 3b.

A 4a: Three Nitzschia cultures from N 1a were inoculated with Asterionella (47,500 cells/flask).

A 4b: As A 4a but nutrient salts added during the experiment.

N 3a: Three Asterionella cultures from A 1a were inoculated with Nitzschia (49,000 cells/flask).

N 3b: As N 3a but nutrient salts added during the experiment.

N 4a=A 4a.

N 4b=A 4b.

Some of the experiments are named by two numbers, e.g. A 3a=N 3a, because in the Figure 2 it is called A 3a when the growth of Asterionella is considered and N 3a when the growth of Nitzschia is considered.

These experiments were running for 7 days. In the b-experiments nutrient salts were added every day, Si in usual quantities and the other salts in a third of the usual quantities. Nutrient salts were added to all cultures in experiment 3 and 4 before inoculation.

The growth rates of the two species in this experiment series were different from the growth rates found in series 1. While in the control (A 1) the divisions/day of Asterionella were 0.65 in series 1, the value was 0.46 in series 2 (A 1a). Nitzschia divided 0.54 times per day during series 1 (N 1) and 0.81 times in series 2 (N 1a). The difference was most likely due to the different illumination and the different temperature in the two series. The smaller growth rate of Asterionella could also be caused by a hereditary character in the new strain.

Asterionella (Figure 2). A 1 shows that addition of nutrient salts during the experiment increases the growth rate. The number of divisions per day was respectively 0.46 (A 1a) and 0.63 (A 1b). In the mixed cultures with both Asterionella and Nitzschia (A 2), Asterionella grew very little and only in one single culture in the experiment without addition of nutrient salts (A 2a); but in A 2b one single culture grew, although more poorly than the
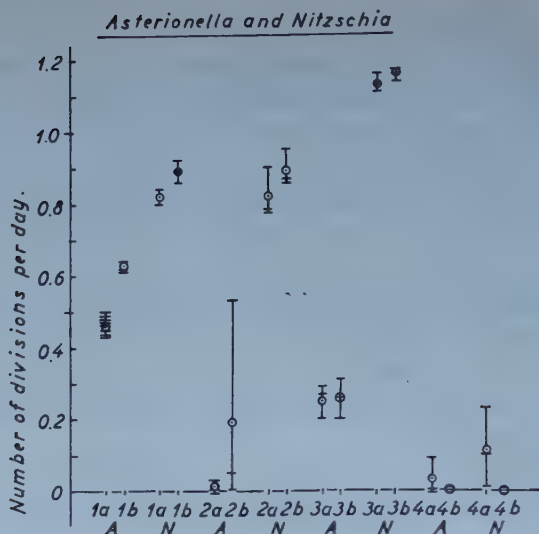
Figure 2. *The number of divisions per day in experiment series* 2. The b-experiments are the same as the corresponding a-experiments, but nutrient salts were added from time to time.

A 1 and N 1. Control with Asterionella and Nitzschia, respectively.

A 2 and N 2. Experiments where Asterionella and Nitzschia were inoculated at the same time.

A 3 and N 3. Nitzschia inoculated in an 8 days old Asterionella culture with living cells.

A 4 and N 4. Asterionella inoculated in an 8 days old Nitzschia culture with living Nitzschia cells.

The lines above A show the number of divisions/day of Asterionella and corresponding N = Nitzschia.

control A 1b, and the other two cultures grew only very little. Thus a distinct inhibition of the growth was found. A great difference is found between this experiment and the corresponding experiment in series 1 (A 2), where the growth of Asterionella was as good as in the control.

In A 3a an 8 days old Asterionella culture was inoculated with Nitzschia cells and was then cultured for 7 days. The divisions/day of Asterionella after the inoculation were 0.25. In A 3b about the same number of divisions per day were found, i.e. 0.26. This indicates that the small growth rate was due to inhibition by substances formed by Nitzschia. The growth rate was of the same order of magnitude in this experiment and in the corresponding experiment in series 1 (A 6). The number of divisions per day was, respectively, 0.25 and 0.28.

In A 4 where an 8 days old Nitzschia culture was inoculated with Asterionella cells no or very little growth took place.

Nitzschia (Figure 2). Addition of nutrient salts during the experiment gave increases in the growth rate as was the case in A 1. In the mixed cultures (N 2a and b) with both Asterionella and Nitzschia, Nitzschia grew as well as in the controls (N 1a and b). Thus no inhibition of the growth was found as was the case in series 1. In an 8 days old culture of Asterionella inoculated with Nitzschia (N 3) this diatom grew very well and better than the control (N 1). The division per day was 0.82 and 0.89, respectively, in the controls (N 1a and b) and in N 3a and b 1.13 and 1.16. This difference cannot be caused by different quantities of nutrient salts in N 1 and N 3 but must be attributed to stimulating substances in the culture solution. In the corresponding experiment in series 1 the results was quite different. There Nitzschia did not grew at all in an 8 days old Asterionella culture.

In N 4, Asterionella was inoculated in an 8 days old Nitzschia culture. The growth rate was somewhat variable in the three single cultures. The number of divisions per day varied from zero to 0.23 during the culturing period. The average number of divisions per day was 0.11. In N 4b no growth was found at all. A comparison with series 1 indicates that the inhibition of the growth of Nitzschia was caused by autotoxic substances.

Experiment series 1 and 2 indicate that both Asterionella and Nitzschia may form substances which inhibit the growth of the other species. The rate of inhibitions seems to depend on the experimental circumstances. While in series 1 Nitzschia does not inhibit the growth of Asterionella a distinct inhibition was found in series 2. As to Nitzschia, a clear inhibition of this species by Asterionella was found in series 1. In series 2 no inhibition of Nitzschia by Asterionella was demonstrated. On the contrary it was suggested that Asterionella may form substances which accelerate the growth of Nitzschia. In series 1 it was similarly suggested that Asterionella may form substances which stimulate the growth of other Asterionella cells. In both series it was found that Nitzschia produce autotoxic substances.

4. Experiments with a diatom in filtrates from cultures of green algae

Experiment series 3. In this series Nitzschia palea was inoculated in filtrates of Scenedesmus quadricauda and Chlorella pyrenoidosa. Scenedesmus and Chlorella were each inoculated in three Drechsel gas washing bottles each containing 100 ml culture solution. A gas mixture containing 5 per cent CO_2 and 95 per cent air was bubbled continuously through the solutions. The bottles were placed in a thermostat bath at $20°C$ and illuminated continuously. Three Drechsel bottles with 100 ml culture solution but not in-
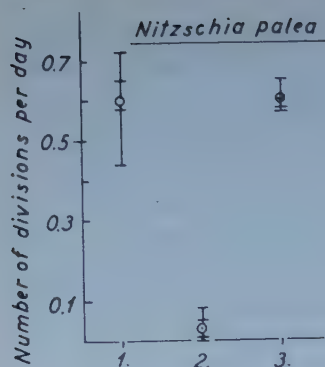
Figure 3. *The number of divisions per day of Nitzschia palea in experiment series 3.*

1. Control.
2. Nitzschia growing in filtrate from a 4—8 days old Scenedesmus quadricauda culture.
3. Nitzschia growing in filtrate from a 4—8 days old Chlorella-pyrenoidosa culture.

The filtrate were added at the start of the experiments and again after 2 and 4 days, respectively.

oculated were set up similarly and were aerated with ordinary air to get a culture solution with the same silica concentration as in the culture solutions with Scenedesmus and Chlorella.

16 polystyrene containers each with 50 ml of culture solution were inoculated the following day with Nitzschia palea (112.000 cells/container). The containers were placed in a thermostat bath at 20°C and illuminated continuously as in series 2.

When the Nitzschia cultures were 3 days old four containers were removed and the cells counted to get the average number of cells in the containers at the start of the main experiment. From the 4 days old cultures of Scenedesmus and Chlorella, respectively, one washing bottle was removed. The Scenedesmus culture was filtered through a glass filter (3 G 3). From the Chlorella culture 100 ml was filtered, after centrifugation, through a collodion filter (membrane filter from Membranfiltergesellschaft, Göttingen). The pH of the Chlorella filtrate was 6.6, of the Scenedesmus 7.5, and of the uninoculated culture solution 8.5. Nutrient salts with N, P and Fe were added to the filtrates in the usual quantities.

From the Nitzschia cultures 25 ml of culture solution was removed. In four of the cultures it was replaced with 25 ml of culture solution from an uninoculated Drechsel flask (control); in four others with 25 ml of Scenedesmus filtrate and in the last four with 25 ml of Chlorella filtrate. The pH of the culture solutions was about 8 in all 12 containers. The Nitzschia cultures were then started again and were kept going for 6 days. The same procedure of the removal of culture solution and addition of filtrate or culture solution from uninoculated Drechsel flasks was repeated 2 and 4 days after the start of the main experiment.

The results are presented in Figure 3. It shows that Nitzschia grew very little in Scenedesmus filtrate. Thus Scenedesmus forms substances which

Figure 4. *The number of divisions per day of Nitzschia palea in experiment series 4.*

1. **Control.**
2. **Nitzschia growing in filtrate from a 13—17 days old Chlorella pyrenoidosa culture.**
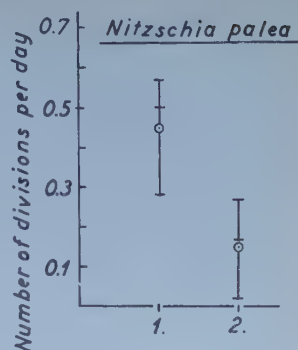
strongly inhibit the growth of Nitzschia. In filtrate from the Chlorella culture Nitzschia grew as well as in the control.

Experiment series 4. It was shown by Pratt that *Chlorella vulgaris* forms autotoxic substances in the culture solution (Pratt and Fong 1940). He showed further that the rate of reduction of the respiration and the photosynthesis in Chlorella cultures growing in filtrates from other Chlorella cultures was dependant on the age of the culture from which the filtrates were made. The reduction was greater in cultures with filtrates from old Chlorella cultures than with those from young ones (Pratt 1943).

Therefore a new experiment series was started where Nitzschia was cultured in filtrate from an older Chlorella culture. The series was carried out in the same way as series 3. The Nitzschia culture was 5 days old when the main experiment with Chlorella filtrate was started. The Chlorella culture was 13 days old. The main experiment continued for 6 days and culture solutions were removed and replaced with filtrate 2 and 4 days after the start as in series 3.

According to Figure 4 the growth of Nitzschia in Chlorella filtrate was distinctly inhibited.

So experiment series 3 and 4 show that both Scenedesmus and Chlorella form substances which inhibit the growth of Nitzschia. In the case of Chlorella the inhibition was obviously dependant on the age of the Chlorella culture.

5. Experiments with green algae in filtrates from a diatom and from green algae

Experiment series 5. 100 ml of Chlorella culture, 100 ml of Scenedesmus culture and 100 ml of uninoculated culture solution (standard culture solution) were prepared in Drechsel bottles in the same way as in series 3.
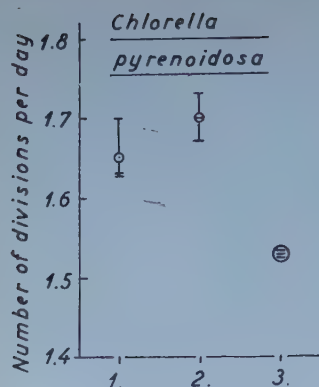
Figure 5. *The number of divisions per day of Chlorella pyrenoidosa in experiment series 5.*

1. Control.
2. Chlorella growing in filtrate from a 21 days old Nitzschia palea culture.
3. Chlorella growing in filtrate from a 21 days old Scenedesmus quadricauda culture.

A Nitzschia culture was made in an Erlenmeyer flask containing 100 ml of culture solution without aeration. After 21 days of culturing, the Chlorella and the Scenedesmus cultures were filtered as described in series 3. The Nitzschia culture was filtered through a glass filter (3 G 3). Nutrient salts were added to the filtrates as in series 3.

The experiments were carried out in test tubes containing 20 ml of culture solution. The test tubes were closed with cotton stoppers and were aerated with air containing 5 per cent CO_2 through glass tubes. The test tubes were placed sloping in a thermostat bath at 20°C and illuminated from below with six tubular fluorescent lamps.

Chlorella. 3 test tubes containing 20 ml of standard culture solution (control), 3 test tubes with 10 ml of standard c. s. and 10 ml of Nitzschia filtrate and 3 test tubes with 10 ml of standard c. s. and 10 ml of Scenedesmus filtrate were inoculated with Chlorella (2.0×10^6 cells/flask). The experiments were kept going for 3 days. The pH in this and the following experiments were about 8 in all culture solutions.

In Figure 5 the results are presented. The growth rate of Chlorella was better in the filtrate from Nitzschia than in the control, while the growth rate in the Scenedesmus filtrate was considerably smaller than in the control. Thus Nitzschia seems to form substances which accelerate the growth of Chlorella, while Scenedesmus forms substances which clearly inhibit the growth of Chlorella.

Scenedesmus. 3 test tubes containing 20 ml of standard culture solution (control), 3 test tubes with 10 ml of standard c. s. and 10 ml of Nitzschia filtrate, 3 test tubes with 10 ml of standard c. s. and 10 ml of Chlorella filtrate and 3 test tubes with 10 ml of standard c. s. and 10 ml of Scenedesmus filtrate were inoculated with Scenedesmus (57,000 cells/flask).

Figure 6 shows the results. While the growth rate of Scenedesmus obviously

Figure 6. *The number of divisions per day of Sce-nedesmus quadricauda in experiment series 5.*

1. Control.
2. Scenedesmus growing in filtrate from a 21 days old Nitzschia palea culture.
3. Scen. growing in filtrate from a 21 days old Chlorella pyrenoidosa culture.
4. Scen. growing in filtrate from a 21 days old Scenedesmus quadricauda culture.
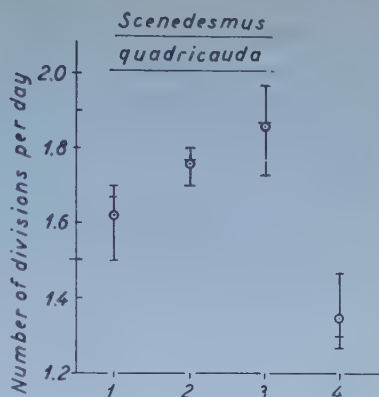
was inhibited in Scenedesmus filtrate it was stimulated in both Nitzschia and Chlorella filtrates.

Thus series 5 shows that Scenedesmus forms autotoxic substances and substances which inhibit the growth of Chlorella, that Chlorella forms substances which stimulate the growth of Scenedesmus, and that Nitzschia forms substances which stimulate the growth of both Chlorella and Scenedesmus.

6. Discussion

Growth inhibiting substances. The production of growth inhibiting substances in algae cultures was first reported by Harder (1917). He found Nostoc punctiforme to form autotoxic substances. Growth inhibiting substances were found also by Pratt (1940) in cultures of Chlorella vulgaris. Several experiments were carried out to study the nature and the effect of the autotoxic substances found in cultures of Chlorella vulgaris (Pratt and Fong 1940; Pratt 1942, 1943, 1944).

An autotoxic substance was found by Levring (1945) in cultures of Skeletonema costatum. Likewise, Nitzschia palea was found by Denffer (1948) to form substances which inhibited the growth of its own cells. The substances appeared to specifically block the mitosis.

In all of the above mentioned investigations the substances studied were able to inhibit the growth of the very same species which produced the substances. Lefevre *et al.* (1949) were able to show the presence in algae cultures of substances inhibiting the growth of other algae. The growth of different algae cultured in filtrates from a Scenedesmus quadricauda culture was more or less inhibited, to a different extent for the different algae. When the same algae species were cultured in filtrates from a Pandorina culture

the growth of some of them was inhibited while that of others was stimulated. Furthermore, the experimental conditions were found to influence the effect of the substances formed in the culture solution. Thus the composition of the medium, the temperature and the age of the culture were all found to play an important rôle.

During the bloom of a single algae species in a pond or another body of water it is commonly observed that other algae species are present in very small numbers. In the canals of Parc Presidentiel de Rambouillet Lefevre *et al.* (1951) observed, at different times blooms of the blue-green algae Aphanozomenon grazile and Oscillatoria planctonica, respectively. Water from the canals was filtered during the period of blooming and the filtrate was inoculated with different algae. The growth of the algae was distinctly inhibited, and the effect was attributed to substances formed in the water by the blooming algae.

Rice (1954) cultivated the algae Chlorella vulgaris and Nitzschia frustulum partly in mixed cultures and partly in filtrates from cultures of the same and of the other species. Both species produced substances which inhibited not only their own growth but also the growth of the other species. When Chlorella and Nitzschia were cultured in filtered water from a pond with a bloom of Pandorina, Rice found the growth of both species to be inhibited.

Very little is known about the nature of the growth-inhibitors. Certain properties were found by Pratt (1942) for Chlorellin, the growth-inhibitor formed by Chlorella vulgaris. In some cases the substances were found to be thermolabile.

All of the algae species studied by the author were found to be able to produce growth inhibiting substances. The investigations mentioned above show further that the production of such substances is a common feature in algae metabolism. The theory advanced by the author as to the inhibition of epiphytic diatoms by the plankton algae thus seems very probable.

A similar theory was presented as early as 1931 by Akehurst. He studied the phenomenon of swarming of plankton algae in certain freshwater ponds during a period of four years. Attempts to correlate the chemical and physical factors necessary for plant growth with rapid development of a single algae species failed for the most part. He concluded that other factors must play a part and suggested that they consisted mainly of a complicated action of certain toxins. His definition of the term toxin was the following: "An excretion product or products of undefined chemical constitution which may also serve as an accessory food and may inhibit or stimulate growth". He divided the plankton algae into two main groups according to food reserves, roughly starch and oil. He suggested that toxic effects do not occur between a species from the oil-group and a species from the starch-group. The toxins of the

oil-group become an accessory food of the starch-group and vice versa. Species from the oil-group stimulated the growth of some species and inhibited the growth of other species of the oil-group. Species from the starch-group stimulated the growth of some species and inhibited the growth of other species of the starch-group. The conclusions of Akehurst are surely too general and not in agreement with all of the results obtained in the experiments mentioned above. The principle of his theory is, however, correct according to all evidence.

Growth stimulating substances. In some of the experiments of Lefevre *et al.* and of the present author, substances were formed which stimulated the growth of the very same species which had produced them, or of other species. The nature of the growth stimulating substances is not known. However, certain organic substances are known to play an important part in the development of algae, e.g., vitamins and various unidentified growth factors in extracts from soil, lake sediments, peat, algae and yeast (see Fogg 1953, p. 84). The growth stimulating substances formed in certain algae cultures are possibly of a similar nature.

Summary

Experiments were carried out with algae in mixed cultures (two species cultures) or with algae cultured in filtrates from cultures of other algae or the same species. In experiments with Asterionella formosa and Nitzschia palea it was shown that both species formed substances which inhibited the growth of the other species and that the rate of inhibition was very dependant on the experimental circumstances. It was shown further that Nitzschia formed autotoxic substances and it was suggested that Asterionella formed substances which accelerated the growth of other Asterionella cells and of Nitzschia in some cases.

In experiments where Nitzschia was cultured in filtrates from Scenedesmus quadricauda and Chlorella pyrenoidosa cultures, it was shown that Scenedesmus formed substances which considerably inhibited the growth of Nitzschia. The growth of Nitzschia was not inhibited in filtrates from a 4—8 days old Chlorella culture but was distinctly so in filtrates from a 13—17 days old Chlorella culture.

When Chlorella was cultured for 3 days in filtrates from a 21 days old Scenedesmus culture a marked inhibition of the growth was found. During 3 days of culturing of Chlorella in filtrate from a 21 days old Nitzschia culture an acceleration of the growth of Chlorella took place.

Similarly an inhibition of the growth was found when Scenedesmus was

cultured for 3 days in filtrates from a 21 days old Scenedesmus culture. On the other hand, an acceleration of the growth of Scenedesmus took place when it was cultured in filtrates from a 21 days old Chlorella and a 21 days old Nitzschia culture, respectively.

References

Akehurst, S. C.: Observations on pond life, with special reference to the possible causation of swarming of phytoplankton. — Jour. Royal Microscop. Soc. 51: 237. 1931.

Denffer, D. v.: Über einen Wachstumshemmstof in alternden Diatomeenkulturen. — Biol. Zbl. 67: 7. 1948.

Fogg, G. E.: The metabolism of algae. — London 1953.

Harder, R.: Ernährungsphysiologische Untersuchungen an Cyanophyceen, hauptsächlich dem endophytischen Nostoc punctiforme. — Z. Bot. 9: 145. 1917.

Jørgensen, E. G.: Silicate assimilation by diatoms. — Physiol. Plant. 6: 301. 1953.

— Variations in the silica content of diatoms. — Ibid. 8: 840. 1955.

— Diatom periodicity and silicate assimilation. — Dansk Bot. Arkiv 16 (in press).

Lefevre, M. & Jakob, H.: Sur quelques propriétés des substances actives tirées des cultures d'Algues d'eau douce. — C. R. (Paris). 229: 234. 1949.

— & Nisbet, M.: Action des substances excretées en culture, par certaines espèces d'Algues, sur le metabolism d'autres espèces d'Algues. — Verh. int. Ver. Limnol. 10: 259. 1949.

— — Compatibilités et antagonismes entre Algues d'eau douce danc les collections d'eau naturelles. — Ibid. 11: 224. 1951.

Levring, T.: Some culture experiments with marine plankton diatoms. — Göteborgs Kungl. Vetenskaps- och Vitterhets-samhälles Handl. 6. Földj. Ser. B. Bd. 3. No: 12. 1945.

Pratt, R.: Influence of the size of the inoculum on the growth of Chlorella vulgaris in freshly prepared culture medium. — Amer. Jour. Bot. 27: 52. 1940.

— Some properties of the growth-inhibitor formed by Chlorella cells. Ibid. 29: 142. 1942.

— Retardation of photosynthesis by a growth-inhibiting substance from Chlorella vulgaris. — Ibid. 30: 32. 1943.

— Influence of the age of the culture on the rates of photosynthesis and respiration. — Ibid. 30: 404. 1943.

— Influence on the growth of Chlorella of continuous removal of Chlorellin from the culture solution. — Ibid. 31: 418. 1944.

— & Fong, J.: Further evidence that Chlorella cells form a growth-inhibiting substance. — Ibid. 27: 431. 1940.

— et. al.: Chlorellin, an antibacterial substance from Chlorella. — Science 99: 351. 1944.

Rice, Theodore R.: Biotic influences affecting population growth of planktonic algae. — Fish. Bull. U.S., No 87, Vol. 54: 227. 1954.

Rodhe, W.: Environmental requirements of fresh-water plankton algae. Experimental studies in the ecology of phytoplankton. — Symb. Bot. Upsaliensis 10: 1. 1948.

Utermöhl, H.: Neue Wege in der quantitativen Erfassung des Planktons. — Verh. int. Limnol. 5: 1931.